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CHARACTERIZATION OF STEROID RECEPTORS OF THE PROSTATE

by

Ⓒ SAUL E. GONOR

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to

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ABSTRACT

To further characterize human prostatic androgen receptor (AR), progesterone receptor (PgR), and estrogen receptor (ER), previously established assay methods were used to quantify cytoplasmic androgen receptor (AR_C), progesterone receptor (PgR_C), and estrogen receptor (ER_C) in a series of 16 prostate cancer specimens. Additionally, the concentrations of nuclear AR (AR_N) in a 0.6 M KCl nuclear extract and in a nuclear matrix preparation were determined for these cancer specimens. Utilizing an hydroxylapatite assay, the androgenic ligand methyltrienolone (R1881), and Scatchard analysis of data, the mean receptor concentrations (fmol/g of tissue ± S.E.M.) observed were: AR_C= 1028 ± 255; PgR_C= 945 ± 253; ER_C= 278 ± 41; AR_N (extractable)= 251 ± 31; AR_N (matrix-bound)= 396 ± 112.

Correlations of disease response with steroid receptor concentrations revealed that AR_C, PgR_C, and ER_C were not useful prognostic indices. However, both KCl-extractable and matrix-bound AR_N were useful prognostic indices. The mean AR_N (extractable) concentrations in those patients with progression of disease or death (n=7), and regression or stabilization of disease (n=9) were 39 ± 15 and 178 ± 38 fmol/g of tissue ± S.E.M., respectively. The mean AR_N (matrix-bound) concentrations in those patients with progression of disease or death, and regression or stabilization of disease were 102 ± 33 and 535 ± 161 fmol/g of tissue ± S.E.M., respectively.

Studies of DNA concentrations in prostate cancer revealed as reported previously, that an extreme variability of DNA concentration per gram of prostate cancer tissue exists. Such variability would seem to preclude expression of ligand binding to AR on a per mg of DNA basis

for this tissue. However, measurement of DNA concentrations allows a correction for the extent of nuclear recovery to be calculated, and is therefore necessary for accuracy in expression of AR_N concentrations on a per g of tissue basis.

Studies designed to characterize ER_C and nuclear ER (ER_N) of benign prostatic hyperplasia (BPH) revealed no ER_N by our methods, and no heat-induced estrogen exchange for ER_C or ER_N.

Studies of modifications of the methods used for tissue pulverization, homogenization, and sedimentation of resulting nuclei through sucrose were performed. These studies revealed that relatively high nuclear recoveries with minimal impurities and maximal AR_N concentrations were obtained by pulverization in liquid nitrogen, homogenization using the Polytron PT-10 homogenizer, and sedimentation of nuclei through 10 ml of 1.8 M sucrose.

Studies of AR_N (extractable and matrix-bound) in crude and purified preparations of BPH nuclei revealed that, following correction for nuclear loss during purification, nuclear purification did not increase detectable AR_N concentrations over the AR_N observed in crude nuclear pellets.

The investigations presented herein further characterize steroid receptors of the prostate, correlate AR_N with disease response in prostate cancer, present a logical basis for expression of ligand binding to AR per g of tissue (corrected for nuclear recovery), and provide refinements in the method of nuclear purification for further study of prostate steroid receptors.

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CHAPTER I

INTRODUCTION

Anatomy and Pathology of the Prostate

The human prostate gland lies immediately inferior to the base of the bladder in the male, where it surrounds the proximal portion of the urethra. The prostate is one of the largest glands in the body with unknown function. It supplies a large number of components to the ejaculate, but the biologic function of the components is unknown (Coffey and Isaacs, 1981).

The prostate is of composite structure, containing fibrous stroma, smooth muscle fibers, and glandular tissue (McNeal, 1981). It is divided into an anterior fibromuscular stroma, which comprises up to one third of the mass of the organ, and a posterior glandular portion, consisting of two fused lobes. Each of these lobes can be subdivided into a central zone of periurethral glands, and a peripheral zone of more laterally placed glands.

Benign nodular hyperplasia (BPH) has been found to be exclusively a disease of the periurethral portion of the gland (McNeal, 1981). This entity results in obstruction of the bladder outlet in up to 75 percent of males over 50 years of age (Wilson, 1980). Management consists of transurethral resection (TURP) of the adenomatous tissue using an electrocautery loop mounted on a panendoscope, or retropubic prostatectomy in cases of large glands.

The other major disease of the prostate is adenocarcinoma, which orginates in the peripheral portion of the gland (Griffiths et al, 1979). In men over age 75, prostate cancer is the second most common malignancy, after lung cancer (Geller, 1979). Depending on patient age and overall medical status, as well as tumour grade and stage, therapy consists of one or a combination of several modalities: TURP, radical prostatectomy with bilateral pelvic lymphadenectomy, radiation therapy (interstitial or external beam), hormonal manipulation, or chemotherapy. In general, hormonal and chemotherapy are reserved for palliation in patients with metastatic disease, while the aforementioned modalities are potentially curative in those patients with localized disease (Geller, 1979).

Endocrine Control of Prostatic Growth

Both the theoretical etiology of benign prostatic hyperplasia and the hormonal therapy of prostatic adenocarcinoma are based upon the principle that growth of the prostate is under androgenic control, and therefore mediated by androgen receptor (AR) (Coffey and Isaacs, 1981). Knowledge of steroid hormone action is therefore essential to the understanding of prostatic disease. In the normal male, the major circulating androgen is testosterone, which is almost exclusively of testicular origin (Coffey and Isaacs, 1981). Luteinizing hormone (LH) is released from the pituitary gland, stimulating Leydig cells to synthesize testosterone from cholesterol. Release of LH is modulated by luteinizing hormone-releasing hormone (LHRH), from the hypothalamus. Furthermore, both the hypothalamus and the pituitary are responsive to negative feedback control from circulating testosterone and/or estrogens (converted from testosterone by peripheral aromatization in the brain to 17β-estradiol).

In the prostate and other sex accessory tissues, testosterone appears to function as a prohormone which is converted by the enzyme 5α-reductase to the active form, dihydrotestosterone (DHT) (Bruchovsky and Wilson, 1968; Coffey and Isaacs, 1981). It is generally accepted that in the absence of testicular androgens, adrenal androgens are insufficient to promote prostatic growth. Indeed, with castration, the prostate involutes and BPH does not develop (Wilson, 1980).

In men over age 50, there is an increase in total plasma estradiol levels of approximately 50 percent, presumably due to an increased peripheral aromatization of testosterone. This increase of estradiol is accompanied by a parallel rise in the plasma protein sex hormone binding globulin (SHBG) (Harper and Griffiths, 1980). Since SHBG preferentially binds testosterone rather than estrogen, the net result is an increase in the free estrogen to free testosterone ratio by as much as 40 percent (Coffey and Isaacs, 1981).

In experiments on dogs it has been demonstrated that the development of BPH is associated with a 3- to 4-fold net increase in DHT concentration within the prostate (Gloyna et al, 1970). However, utilizing physiologic replacement doses of either testosterone or DHT, investigators were unable to induce the development of prostatic growth in castrated dogs comparable to that in intact control animals (Wilson et al, 1975). Subsequently, the administration of 3α-androstanediol in combination with small amounts of 17β-estradiol to the castrated dog resulted in profound prostatic enlargement (Walsh and Wilson, 1976). It was then demonstrated that 3α-androstanediol acts a precursor of DHT (Moore et al, 1979). This in turn is associated with the development of BPH in this animal model (Isaacs and Coffey, 1981).

Human prostate also exhibits an increase in DHT in hyperplastic tissue (Wilson, 1980) suggesting that estrogens could play a major role in the etiology of human BPH. It has further been shown that estrogen enhances the level of AR within the prostate gland of dogs, which allows for androgen-mediated growth even in the face of declining androgen production with advancing age (Wilson, 1980). The mechanism by which estrogen increase prostate AR concentrations is unknown, but is thought to be due to a direct action of estrogen on the prostate cell (Wilson, 1980).

In rats, prolactin has been shown to be synergistic with androgens in promoting prostatic growth, and prolactin receptors have been identified in prostatic tissue (Coffey and Isaacs, 1981). Furthermore, in humans, hyperplastic prostates have been shown to contain elevated prolactin levels (Ron et al, 1981), but the significance of this is unresolved. Prolactin may accentuate the effect of androgens in stimulating prostatic growth (Griffiths et al, 1979).

The observation that both glandular and stromal portions of the gland proliferate in BPH (Wilson, 1980; Rohr et al, 1980) is important in the evaluation of investigations based on biopsy samples, which may contain predominantly glandular or stromal tissue.

Approximately 60 to 80 percent of prostatic cancers are under androgenic control (Shain and Boesel, 1978; Geller et al, 1981; Trachtenberg et al, 1981). It has long been realized that androgen ablation by orchidectomy will therefore reduce the growth of these tumours. For the past 40 years, estrogen therapy has also been used to suppress testosterone production to castrate levels, by its indirect action of inhibition of LH release (Hodges, 1979). Whether estrogen also acts directly on the prostate, by means of receptors, is unknown.

Medical or surgical adrenalectomy and/or hypophysectomy cause subjective and some objective improvement of disease status in 35 to 50 percent of patients in relapse because of failure of primary endocrine therapy (Hodges, 1979).

Despite the well recognized benefit of endocrine manipulation, orchidectomy is unacceptable to many patients, and estrogen therapy carries with it complications of feminization and thromboembolic disease (Veterns Administration Co-operative Urological Research Group, 1967; Geller, 1979). Furthermore, the common practice of withholding hormonal therapy until such time as the patient is symptomatic may lead to a reduction in efficacy of other adjuvant modalities (Trachtenberg and Walsh, 1982). It has recently been demonstrated that patients with androgen-insensitive prostate carcinoma have significantly longer survival on chemotherapy if the protocol is inititiated within one year of diagnosis (Paulson, 1983). Therefore, the use of hormonal manipulation must be judiciously reserved for those patients whose tumours are truly androgen-dependent. An index capable of predicting hormonal responsiveness could be of great benefit in selecting those patients best managed by endocrine manipulation, and those who should receive chemotherapy at a time when their tumour burden is less, in the hope that this would increase both patient tolerance and tumour response.

For those patients with hormone-dependent tumours, new therapeutic agents have been developed recently. Pharmacologic doses of LHRH have a paradoxical effect of reducing LH release and Leydig cell responsiveness to LH (Jacobi and Wenderoth, 1982) and recent trials with long-acting LHRH analogues have shown these agents to be efficacious in reducing serum testosterone values to castrate levels, without the adverse effects of estrogen therapy (Jacobi and Wendertoth, 1982; Borgman et al, 1982). Other potential means of hormonal manipulation include the anti-androgen, cyproterone acetate, which may act through progesterone receptors, flutamide, a non-steroidal compound (Mainwaring, 1979), and megestrol acetate (Geller et al, 1981). In order to adequately compare these agents to the established methods of hormonal therapy, prospective trials in patients with proven hormone-dependent tumours are mandatory.

Steroid Hormone Physiology

Since the prostate is under androgenic control, it is considered to be a steroid hormone-dependent organ. All steroids act through similar pathways to produce the same general effect of mRNA induction and protein synthesis. These effects are mediated by specific, high affinity hormone binding proteins termed receptors (Grody et al, 1982). Through the use of radiolabelled steroids, cytoplasmic receptors have been demonstrated in a variety of target tissues. Each receptor binds to its respective hormone with great specificity and high affinity, and is present in significant amounts only in target tissue cells (Grody et al, 1982). However, the means by which the steroid enters the cell and the exact nature of hormone-receptor interaction remain unresolved (Baxter and Funder, 1979). Following hormone binding, the receptor undergoes a conformational or enzymatic activation called transformation, which enables it to translocate into the nucleus (Grody et al, 1982). Once in the nucleus, the hormone interacts with the genome, resulting in mRNA induction and subsequent protein sythesis. This occurs by way of as yet hypothetical nuclear acceptor sites, felt to be specific areas of chromatin-associated non-histone proteins (Barrack and Coffey, 1982). The fate of receptors after binding to these acceptor sites is completely unknown (Grody et al, 1982) although they may cycle back to the cytoplasm.

Nuclear Steroid Hormone Action

That nuclear binding of hormone is necessary for protein induction has been demonstrated, at least in the case of estrogen receptor (ER), by the positive correlation of concentrations of nuclear hormone-receptor complex with "estrogen-induced protein" (IP) concentrations in the rat (Galand et al, 1978). Other animal experiments have demonstrated that sustained presence of estrogen in the nucleus is necessary for DNA sythesis (Gorski et al, 1977). Studies of these nuclear steroid-binding sites have revealed that only a limited number of the nuclear sites is necessary for estrogenic induction of maximal uterine growth in the rat (Anderson et al, 1973). That is, although certain target cells for a sex-steroid hormone contain up to 10,000-20,000 cytoplasmic receptors for that steroid, full physiologic response is seen when only about 2000 of these hormone-receptor complexes interact with the nucleus (Clark and Peck, 1976; Leake, 1981). Although traditionally it has been claimed that unbound receptor exists only in the cytoplasm, recent autoradiographic work suggests that free receptor may be present in the nucleus as well, indicating that unbound receptor may be in equilibrium between the two compartments (Martin and Sheridan, 1982).

Much study has been devoted to characterization of nuclear acceptor sites. The nuclear matrix has been demonstrated to be a residual nuclear skeleton following treatment with detergent, DNase, RNase, and high salt concentrations (Berezney and Coffey, 1977; Kaufmann et al, 1981). This matrix consists of a residual nuclear lamina, highly condensed residual nucleoli, and an extensive granular and fibrous interchromatinic scaffold structure which extends throughout the nucleus, and provides functional organization for the DNA (Barrack and Coffey, 1982). The multiple biological functions associated with the nuclear matrix have been reviewed recently (Maul, 1982) and include sites for DNA and RNA synthesis (Berezney and Bucholtz, 1981), and sites of nuclear acceptors of steroid hormones (Barrack and Coffey, 1982).

Prior to demonstration of the nuclear matrix, labelled receptors which could be extracted from nuclei by high salt concentration (0.6 M KCl) were thought to represent the major component of nuclear hormone action. However, it now appears that a proportion of nuclear receptors are bound to the nuclear matrix, are therefore salt-insoluble (Ruh and Baudendistel, 1977), and may be the major determinants of steroid hormone action (Clark and Peck, 1976; Barrack and Coffey, 1980 and 1982; Swanek et al, 1982). Barrack and Coffey (1980) have emphasized that the concentration of matrix-associated binding sites changes in response to manipulation of the hormonal status of the animal studied. In addition, Swanek et al (1982), demonstrated a 15- to 20-fold increase in the number of matrix-bound ER sites within 68 hours of induced prostatic growth in the rat. Furthermore, dexamethasone-sensitive clones of murine leukemic myeloblasts contain nuclear salt-resistant glucocorticoid receptor, which is not present in dexamethasone-resistant clones. Nuclear salt-resistant receptors may therefore be involved in the hormonal dependence of neoplastic cells as well (Barrack et al, 1983).

Type I and Type II Estrogen Receptors

A further area of investigation and controversy in steroid hormone action is the probable heterogeneity of the estrogen receptor. The classical ER has a low capacity with high affinity, and has been designated as type I. A second type of receptor has been described, with a large capacity for steroid binding, but with low affinity, and has been termed type II (Clark et al, 1978; Eriksson et al, 1978). This type II estrogen binder has been described for rat uteri (Reichman and Villee, 1978; Markaverich et al, 1980; Markaverich et al, 1981a), human mammary tumours (Panko and Clark, 1981; Panko et al, 1981), as well as various other estrogen-dependent tissues (Markaverich et al, 1981a). Initially it was theorized that the type II sites were actually alpha-fetoprotein (Labarbera and Linkie, 1978), but this has subsequently been refuted (Clark et al, 1978; Kiang et al, 1978; Nakao et al, 1978).

Type II sites have been described in both the cytoplasm (Clark et al, 1978) and nucleus (Markaverich et al, 1980) of rat uteri. Cytoplasmic type II sites may represent precursors of type I sites (Clark et al, 1978), or may bind excess estrogen, allowing it to be concentrated within the cell (Clark and Markaverich, 1981). Cytoplasmic type II sites are distinct from nuclear type II sites, which are activated by the binding of estrogen-receptor complex in the nucleus (Markaverich et al, 1980). The function of the nuclear type II sites is also unknown, and they may interfere with measurement of type I sites in clinical assays (Markaverich et al, 1980).

Because of the relatively low affinity of cytoplasmic and nuclear type II sites, high steroid concentrations are required for their demonstration. Cytoplasmic and nuclear type II sites will produce a two-slope pattern on Scatchard analysis (Panko et al, 1981), allowing their detection and quantification. Furthermore, nuclear type II sites can be inactivated by the sulfhydryl-reducing agent DTT, and are not bound by Nafoxidine, allowing their separation from nuclear type I sites (Markaverich et al, 1981a and b; Clark et al, 1982).

The long term retention of estradiol by the nucleus, which is required for a hormonal effect, has been shown to be associated with stimulation of nuclear type II sites (Clark and Markaverich, 1981b; Clark et al, 1982). It has therefore been suggested that these sites are chromatin-associated and/or matrix-bound (Clark and Markaverich, 1981b), and may "process" the type I sites prior to regulation of transcriptional events and subsequent uterine growth (Markaverich et al, 1980). Furthermore, uterine growth correlates more closely with levels of type II than type I sites (Markaverich et al, 1980), and inhibition of uterine growth by dexamethasone is associated with selective inhibition of type II sites (Markaverich et al, 1980; Clark and Markaverich, 1981). These receptors are distinct from those unfilled nuclear sites, demonstrated in immature rat uteri, which have a binding affinity similar to that of the classical estrogen receptor, and a unique sedimentation coefficient (Carlson and Gorski, 1980).

Heterogeneity in hormone binding sites has also been observed for glucocorticoids and progesterone, suggesting that this may be a general phenomenon (Clark and Markaverich, 1981). The possibility that both types of ER might be involved in estrogenic control of prostatic growth has yet to be investigated.

Assays for Prostatic Steroid Receptors

In addition to the aforementioned complexities in steroid hormone receptor physiology, a further level of complexity arises from the various available means of receptor quantification. Assay techniques for steroid hormones are numerous, and the technical aspects of these are beyond the scope of this review. However, one point of sufficient importance to merit inclusion is that of sodium molybdate. This agent has proved very useful in many investigations, in a variety of tissues, as a cytoplasmic steroid receptor stabilizer (Neilsen et al, 1977; McBlain and Shyamala, 1980; Nishigori and Toft, 1980; Noma et al, 1980; Hawkins et al, 1981; Trachtenberg et al, 1981; Grody et al, 1982; Sirett and Grant, 1982; Tsai and Steinberger, 1982). It has the additional feature of preventing transformation of steroid-bound receptor (Nishigori and Toft, 1980; McBlain et al, 1981; Grody et al, 1982). Both of these properties enable it to greatly enhance quantification of cytoplasmic receptor (McBlain and Shyamala, 1980; Noma et al, 1980; Trachtenberg et al, 1981; Sirett and Grant, 1982; Tsai and Steinberger, 1982), but its effect on nuclear matrix-bound receptors has not been studied to date. However, it has been suggested that molybdate may extract steroid receptors from nuclei if the molybdate is present in the tissue homogenization buffer (Trachtenberg et al, 1981).

Development of steroid assays for the human prostate has been hampered by the relative instability of AR, as well as tissue contamination by sex hormone-binding globulin (SHBG), which binds testosterone and DHT with similar affinity to that of AR (Trachtenberg et al, 1981; Ekman, 1982). Natural ligands, such as DHT, are of limited value due to their rapid metabolism even at low temperatures (Ekman, 1982), but synthetic steroids have improved the reliability of these assays. Methyltrienolone (R1881) is a synthetic androgen which binds to AR but not to SHBG (Shain and Boesel, 1978), is also resistant to metabolic conversion, and exchanges with receptor-bound endogenous DHT to about 70 per cent during overnight incubation at 0°C (Menon et al, 1978). However, R1881 also binds to progesterone receptor (PgR). To improve specificity, a 1000-fold excess of triamcinolone (TA) is added to the assay in order to occupy any PgR which may be present (Trachtenberg et al, 1982; Donnelly, 1982; Ekman, 1982). In addition to AR, ER and PgR may also be measured in the prostate, using the radioligands 17β-estradiol and promegestone (R5020) respectively. The assay has been further refined by using an hydroxylapatite assay, which may be superior to the dextran-coated charcoal assay at lower protein concentrations (Trachtenberg et al, 1981; Donnelly, 1982; Ekman, 1982), or in the presence of endogenous protease enzymes. In addition to molybdate, phenylmethylsulfonylfluoride (PMSF) is used to inhibit proteases by binding to serine residues (Trachtenberg et al, 1982).

Using various modifications of these assay techniques, numerous investigators have measured receptor levels in normal, hyperplastic, and malignant prostates. Initially cytoplasmic and more recently salt-extractable and salt-resistant nuclear receptors have been quantified, as summarized below.

Receptors in Benign Prostatic Hyperplasia

In investigations of the etiology of BPH, various receptors have been determined by single point assay (one receptor-saturating steroid concentration) or microassay (Hicks and Walsh, 1979) and by Scatchard analysis of data from assays using multiple steroid concentrations (Ekman, 1982; Trachtenberg et al, 1982). Cytoplasmic AR (AR_C) is uniformly present in high concentration, comparable to that of normal (non-hyperplastic) specimens (Donnelly, 1982; Ekman, 1982; Trachtenberg et al, 1982). Nuclear AR (AR_N) is consistently present in BPH in salt-extractable (Trachtenberg et al, 1981 and 1982; Barrack et al, 1983; Donnelly, 1982; Shain et al, 1982), salt-resistant (Barrack et al, 1983), and matrix-bound fractions (Donnelly, 1982). The ratio of salt-extractable to salt-resistant or matrix-bound AR_N in BPH varies from 1:1 (Barrack et al, 1983) to 1:2 (Donnelly, 1982).

Cytoplasmic PgR (PgR_C) is also present in most, if not all, BPH specimens, although in lesser quantities than AR (Martelli et al, 1980; Donnelly, 1982). The function of PgR in BPH is unknown (Coffey and Isaacs, 1981). Since PgR_C is less frequently found in normal tissue than BPH, it has been speculated that PgR-positive "normal" tissue actually represents early hyperplasia (Ekman, 1982). Alternatively, PgR levels may reflect estrogen activity, as in the breast (McGuire, 1980).

Results of cytoplasmic ER (ER_C) determinations in BPH vary widely (Murphy et al, 1980; Ekman, 1981; Auf and Ghanadian, 1982; Pontes et al, 1982; Donnelly et al, 1983). In normal specimens, ER_C has been found in low concentrations (Murphy et al, 1980; Donnelly et al, 1983). In BPH, ER_C originally was uniformly demonstrated only using single point assays, which are inaccurate at low receptor concentrations or under non-equilibrium conditions (Ekman, 1982). However, using Scatchard analysis, it appeared that ER was not uniformly present in both cytosol and nuclear-extractable fractions of BPH (Ekman et al, 1979a; Murphy et al, 1980; Ekman, 1981 and 1982). These results tended to refute the proposed role of estrogen in the pathogenesis of BPH. However, these investigators did not use the receptor stabilizer sodium molybdate or the protease inhibitor PMSF in their preparations. Molybdate is known to increase both measureable AR_C and PgR_C levels in BPH (Sirett and Grant, 1982). Recent studies in which molybdate was used report that most BPH specimens were ER-positive in the cytoplasmic (Murphy et al, 1980; Auf and Ghanadian, 1982; Donnelly et al, 1983; Pontes et al, 1982) and nuclear-extractable (Auf and Ghanadian, 1982) fractions.

Non extractable nuclear ER (ER_N) has not yet been quantified in BPH specimens. Furthermore, types I and II ER_N have been described in the rat prostate (Swanek et al, 1982), suggesting a further level of possible estrogen activity in prostatic growth, although these have not yet been detected in the human prostate.

Receptors in Adenocarcinoma of the Prostate

One possible measurement of the endocrine sensitivity of a malignancy is the presence of an appropriate steroid receptor within the tumour. If the presence of significant levels of receptor is found to correlate with response to hormonal manipulation, this modality may be used more selectively and hopefully with greater response. Based on the established predictive value of ER_C in breast cancer (McGuire, 1980; Mobbs, 1982), AR_C, PgR_C, and ER_C plus salt-extractable AR_N have been measured in prostatic adenocarcinoma (Trachtenberg et al, 1981; Ekman, 1982) and the receptor profile appears more variable than that seen with BPH (Ekman, 1982).

AR_C is present in most cancer specimens (Ekman et al, 1979b; Shain et al, 1980; Trachtenberg et al, 1981; Ekman, 1982), and PgR_C is present in approximately 50 percent of specimens (Ekman, 1981 and 1982; Trachtenberg et al, 1981). ER_C has been reported as absent (Ekman, 1981), or present in low concentrations in the majority of specimens (Trachtenberg, 1981). AR_C concentrations have been correlated with patient survival, with variable results (de Voogt and Dingjan, 1978; Wagner and Schulze, 1978; Ekman et al, 1979b; Martelli et al, 1980; de Vere White and Olsson, 1981; Ekman, 1982) and the current consensus is that AR_C does not correlate with therapeutic response (Martelli et al, 1980; de Vere White and Olsson, 1981; Ekman, 1982). It has therefore been suggested that AR_N levels may provide a better prognostic index (de Vere White and Olsson, 1981; Ekman, 1982) and two recent reports suggest that a correlation may exist between nuclear-extractable AR and hormone responsiveness in patients with prostatic cancer (Mohla et al, 1982; Trachtenberg and Walsh, 1982).

There is accumulating evidence that the nuclear matrix-bound steroid receptor (or acceptor) sites are the biologically important receptor sites for steroid action (Barrack and Coffey, 1982). Therefore, as an index of hormonal dependence of target tissues, the measurement of nuclear matrix-bound receptors should be superior to assays of either cytoplasmic receptors, or those receptors which can be extracted from the nucleus by high salt concentrations.

Recently Barrack et al (1983) have assayed the non-extractable fraction of AR_N in BPH and prostatic cancer using crude nuclear preparations. Although these preparations would have contained cytoplasmic contaminants in addition to nuclear matrices, a significant percentage of the AR_N was associated with the salt-resistant fraction. However, relatively pure human prostate cancer nuclear matrices have not been isolated to date, and thus matrix-bound AR_N has not been assayed specifically in this tissue.

It is too optimistic to believe that receptor assays will become the primary means of estimating the pathophysiologic activity of prostatic cancer. Rather, these studies should be regarded as a valuable complement to present diagnostic modalities in individualizing the approach to the treatment of this tumour (Ekman, 1982). Perhaps the introduction of radioimmunoassay for steroid receptors, based on purification of monoclonal antibodies to isolated receptor, will enable a safe, reproducible, routine method of receptor assay to be developed (Ekman, 1981). However, without receptor purification this cannot be accomplished. Furthermore, current assay methods still require considerable refinement prior to introduction into clinical practice. Methods of nuclear purification are inadequately documented, and the validity of nuclear receptor quantification in the presence of cytoplasmic contamination has not been investigated. Correlations between concentrations of matrix-bound AR_N and patient survival in prostate cancer have not been reported, and the role of ER_N in the etiology of BPH is unknown.

Research Proposals

In order to further characterize steroid receptors in the prostate gland in the hope of further elucidation of both the etiology of BPH and androgenic dependency of prostate cancer, the following studies were undertaken during the course of this project:

1) Quantification of AR_C, PgR_C, ER_C, extractable and matrix-bound AR_N, in a series of patients with metastatic adenocarcinoma of the

prostate.

2) Correlation of AR concentrations in the various subcellular compartments with objective response to endocrine therapy in patients with adenocarcinoma of the prostate.
3) Improvements in methods of nuclear purification in prostate specimens.
4) Quantification of AR_N in crude and purified prostatic nuclear preparations, in order to determine whether nuclear purification reveals additional binding sites.
5) Utilization of an estrogen exchange assay to reveal any endogenously-bound ER in the cytoplasm and nuclear matrix of the prostate.
6) Quantification of extractable and matrix bound ER_N of the normal and hyperplastic prostate.
7) Quantification of cytoplasmic type I and type II estrogen binding sites in the rat uterus, as a preliminary model for investigation of multiple estrogen binding sites in the human prostate.

CHAPTER II

METHODS

Patients

Normal prostate tissue was obtained by total prostatectomy of 3 cadaveric renal transplant donors (ages 10, 34, and 40 years).

Benign prostatic hyperplastic tissue (BPH) was obtained by retropubic prostatectomy of 11 patients with symptomatic prostatic obstruction.

Fifteen patients with metastatic (n=12) or locally invasive (n=3) adenocarcinoma of the prostate who underwent TURP for urinary obstruction secondary to malignancy were the source of prostatic adenocarcinoma (tissue was obtained as described below). A tumour-replaced pelvic lymph node was obtained at the time of staging pelvic lymphadenectomy for adenocarcinoma of the prostate in one additional patient.

As shown in Appendix 2, all 16 cancer patients were staged clinically by history and physical examination (including rectal examination), serum acid phosphatase by enzymatic assay, radionuclide bone scan, plus radiographic studies in selected cases. All patients were weighed, and had hemoglobin measured pre-operatively. Twelve of the 16 patients had a positive bone scan, 11 had elevation of the serum acid phosphatase concentration greater than 0.8 IU/L, and 9 patients had both an abnormal bone scan and an elevated acid phosphatase determination. None of the 16 adenocarcinoma patients had received any endocrine therapy prior to entry into the study. However, 3 patients had received external beam pelvic radiotherapy for their malignancy more than four years prior to entry into the study and had since had recurrence of disease. Following surgery, all patients received either estrogen therapy consisting of an oral dose of 3 mg of diethylstilbestrol (DES) once daily (n=11), or orchidectomy (n=5) (see Appendix 3).

Patient Follow-up (Adenocarcinoma of the Prostate)

Six months following initiation of hormonal therapy, patients were recalled for assessment of disease regression or progression, according to the National Prostatic Cancer Project response criteria (see Appendix 1). This review assessment included an interview to assess compliance with respect to medications, bone pain, and anorexia. Patients were weighed and hemoglobin and serum acid phosphatase were determined. Radionuclide bone scans and in selected cases, supplementary radiographs, were also obtained as shown in Appendix 3. Results of the post-treatment review assessment were then compared to the initial pre-treatment assessment, and the patient was placed into 1 of 5 categories of disease response, according to the criteria shown in Appendix 1: objective complete response, objective partial response, objective stabilization of disease, objective progression, or death. The details of this disease response classification are shown in Appendix 1, and for each patient the disease response was correlated with the results of the receptor studies described below.

Animals

For experiments concerning type I and type II estrogen receptors (Clark et al, 1978), immature rat uteri were obtained from 5-6 week old female Sprague-Dawley rats (Biosciences Animal Services, University of

Alberta). Animals were kept in a controlled environment, with the temperature maintained at 21°C, and a lighting cycle of 12 hours of light: 12 hours of darkness. Food and water were provided *ad libitum*. For some experiments, the animals received 5 μg 17β-estradiol in 5% ethanol (v/v) in normal saline, injected intraperitoneally using a 22 gauge needle, 1 hour prior to sacrifice. Animals were sacrificed by ether asphyxiation and laparotomized immediately. Uteri were removed, stripped of connective tissue, blotted, placed on ice and transported to the laboratory. They were then immersed in liquid nitrogen for rapid freezing, and stored at -70°C until assayed.

Prostate Tissue Specimens

Normal and benign hyperplastic prostate specimens, all obtained by open surgery, were stripped of connective tissue and chopped into small portions immediately. Other investigators have used the peripheral zone of prostate tissue (surgical capsule) from radical cystoprostatectomy specimens (Barrack et al, 1983) as a source of normal tissue. However, we have avoided this peripheral tissue, because the patients from which it could be obtained were generally over 40 years of age, and therefore their prostates have been subjected to a changing hormonal milieu, which could influence the steroid receptor concentrations (Wilson, 1980). Furthermore, in the presence of periurethral adenoma, the surgical capsule can no longer be considered to be normal prostate, but rather a compressed atrophic remnant of the normal gland, with areas of atypical hyperplasia (McNeal, 1981).

Fifteen malignant prostate specimens were obtained using a Thompson cold punch resectoscope or cold knife at the time of TURP, and 1 specimen consisted of tumour-replaced lymph node obtained at the time of staging pelvic lymphadenectomy. A small sample of each specimen was sent for histologic confirmation of malignancy and was positive in all cases. Needle biopsy specimens do not provide sufficient amounts of tissue required for nuclear preparations and receptor quantification by Scatchard analysis (see below), and TURP specimens are unsuitable because the electrocautery denatures receptor proteins (Donnelly, 1982; Kitano et al, 1983). Although others have used radical prostatectomy specimens as a source of malignant tissue (Barrack et al, 1983), in our experience, specimens obtained by this method are too small (less than 1 g, as described below) to be of value. Furthermore, it is often very difficult to be confident that all of the selected tissue is malignant, because of distortion of the consistency of the normal tissue secondary to the previous biopsy and surgical trauma.

All specimens were transported on ice to the laboratory, immersed in liquid nitrogen, and then stored at -70°C until the time of assay.

Steroids

R1881 (methyltrienolone, [17-methyl-^{3}H] 17β-hydroxy-17-methyl-estra-4,9,11-trien-3-one), specific activity (s.a.) 87 Ci/mmol, R5020 (promegestone [17-methyl-^{3}H] 17α, 21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione), s.a. 87 Ci/mmol, and unlabelled R1881 and R5020 were obtained from New England Nuclear, Boston, Mass. Estradiol-17β ([2,4,6,7-^{3}H] estra-1,3,5 (10)-triene-3, 17β-diol), s.a. range 92-114 Ci/mmol, was obtained from Amersham, Montreal, Quebec. Unlabelled diethylstilboestrol (DES), triamcinolone acetonide (TA), dexamethasone, progesterone, 17β-estradiol and dihydrotestosterone (DHT), were obtained from Sigma Chemical Co., St. Louis, Missouri.

Other Materials

Hydroxylapatite (HAP) and Bio·Rad protein assay kits were obtained from Bio·Rad Laboratories, Richmond, California. Phenylmethylsulfonylfluoride (PMSF), bovine serum albumin, TRIS, monothioglycerol (MTG), calf thymus DNA, diphenylamine reagent, and charcoal were obtained from Sigma Chemical Co., St. Louis, Missouri. Sodium molybdate, magnesium sulfate, sodium phosphate (dibasic), potassium chloride, sodium hydroxide, trichloroacetic acid, perchloric acid, acetaldehyde, 1-propanol, and glacial acetic acid were all of reagent grade and obtained from Fisher Scientific Co., Quebec. Triton X-100 was obtained from BDH Chemicals, Toronto, Ontario. Sodium tetrathionate (NaTT) was obtained from Fluka Chemical Corp., Hauppauge, N.Y. Deoxyribonuclease (DNase I) (2333 units/mg) was obtained from Millipore Corp., Freehold, N.J. Sucrose was obtained from Schwartz/Mann, Spring Valley, New York. Ethanol (95%) was obtained from Stanchem, Winnipeg, Manitoba. Ready-Solv HP scintillation fluid was obtained from Beckman Instruments, Fullerton, California. Double distilled water was used to prepare appropriate reagents.

Preparation of Cytosol

Tissue stored at -70°C was thawed on ice, and all procedures were performed at 0-4°C. Approximately 125 mg of tissue were required for each 9 point Scatchard analysis (see below). The tissue was weighed, chopped into fine portions on dry ice with a razor blade, and homogenized in TM-PMSF buffer (10 mM Tris, 12 mM MTG, 10 mM sodium molybdate, 10% (v/v) glycerol, 1 mM PMSF, pH 7.4 at 22°C) with a tissue: buffer ratio of 125 mg : 2 ml, using a Polytron PT-10 homogenizer (Brinkmann), set at 4 (four 5 second homogenizations with 30 second intervening cooling periods). The homogenate was then centrifuged in a Beckman L2-65B ultracentrifuge at 200,000 x g for 30 minutes, using an SW60 Ti rotor, to yield the supernatant cytosol fraction.

Preparation of Nuclear Matrices

Approximately 1 g of prostate tissue was weighed, immersed in liquid nitrogen, and pulverized using either a mortar and pestle or a thermovac pulverizer (Thermovac, Copiague, N.Y.). Using the pulverizer gun, 15-20 actions were required to fracture the tissue to a powder grossly approximating the consistency of that obtained with the pestle technique. All subsequent procedures were performed at 0-4°C. In all malignant specimens, the pulverized tissue was suspended in 15 ml of STM-PMSF buffer (0.25 M sucrose, 50 mM TRIS, 5 mM $MgSO_4$, 1 mM PMSF, pH 7.6 at 22°C) and homogenized with an all glass Tri-R SGS/75 tapered tissue homogenizer (Tri-R Instruments Inc., Rockville Centre, N.Y.), requiring approximately 40 strokes over 3 minutes, on ice, with intermittent cooling periods. In later experiments (described below) the tissue was not homogenized in the glass homogenizer, but was homogenized using the Polytron PT-10 homogenizer as described above.

The homogenate was centrifuged at 800 x g for 10 minutes, the supernatant containing extracellular debris and blood products was discarded, and the crude pellet was resuspended in 15 ml STM-PMSF, to which 10 μl of Triton X-100 was added (final concentration of 0.1%). Following 10 minutes of the detergent treatment, the specimen was centrifuged at 800 x g for 10 minutes, and the supernatant discarded. The pellet was resuspended in 15 ml of STM-PMSF, and filtered through a 30 mesh wire screen, for preliminary nuclear purification. Following a third centrifugation at 800 x g for 10 minutes, the supernatant was discarded, the pellet was resuspended in 25 ml STM-PMSF, carefully layered over 5 ml of 1.8 M sucrose (this step was also varied in later experiments), and centrifuged at 74,000 x g for 30 minutes, using a Beckmann SW28 Rotor in a Beckmann L2-65B Ultracentrifuge, to yield purified nuclei.

The purified nuclear pellet was suspended in 2 ml of 0.6 M KCl, incubated for 30 minutes, and centrifuged at 10,000 x g for 10 minutes. Following a second 0.6 M KCl incubation for 15 minutes and centrifugation at 10,000 x g for 10 minutes, the supernatants were pooled and designated the first KCl extract. The pellet was incubated with 2 ml of STM-PMSF containing DNase I (100-500 IU/ml final concentration) for 1 hour, then centrifuged at 10,000 x g for 10 minutes. The supernatant was discarded, the pellet resuspended in 2 ml of 0.6 M KCl, incubated for 15 minutes, and centrifuged at 10,000 x g for 10 minutes, to yield the second KCl extract. The final pellet, containing nuclear matrices, was resuspended in 2 ml of STM-PMSF buffer. Since it has been previously demonstrated (Donnelly, 1982) that these methods successfully isolate the nuclear matrix with a relatively high degree of purity, we have referred to the salt-resistant or non-extractable binding sites as matrix-bound receptor.

Steroid Binding Assays

In all cases, 200 μl of cytosol, nuclear extract, or nuclear matrices were added to 0.5 ml of an hydroxylapatite (HAP) suspension [0.1 g HAP/ml TNP buffer (50 mM Tris, 10 mM sodium phosphate, pH 7.4 at 22°C)] plus 0.5 ml TNP buffer, and shaken for 60 minutes to bind the receptor to the HAP. Following this, the HAP suspension was centrifuged at 12,800 x g (Eppendorf 5412 microcentrifuge) for two minutes and the supernatant discarded. Serial dilutions of steroids were added to the residual pellet, the pellet was resuspended and incubated at 4°C.

A saturation analysis with tritiated ligand in six concentrations (0.2-10 nM for nuclear preparations, and 0.1-5 nM for cytosol preparations) was used to measure total binding, and tritiated ligand in the presence of a 100-fold excess of unlabelled steroid was used to measure nonspecific binding at 3 concentrations (see Appendix 6). [^{3}H]R1881 in the presence of a 1000-fold excess of TA, with or without a 100-fold excess of unlabelled R1881, was used for the AR binding assays. [^{3}H]R5020 in the presence of a 10-fold excess of both dexamethasone and DHT, with or without a 100-fold excess of unlabelled R5020, was used for the PgR binding assays. [^{3}H]17β-estradiol, with or without a 100-fold excess of DES, was used for the ER binding assays.

For the experiments on type I and type II ER, an expanded set of dilutions was used to provide 12 concentrations of tritiated estradiol for total binding (0.1-40 nM final concentration), and 4 concentrations of tritiated estradiol in the presence of a 100-fold excess of DES for nonspecific binding as shown in Appendix 7.

Following incubation of HAP-bound receptors with steroids for 16-20 hours, unbound steroid was removed from the HAP by four washes of 10 mM phosphate buffer as follows: the HAP pellet was suspended in 1 ml of the phosphate buffer and shaken for five minutes; following centrifugation for two minutes at 12,800 x g the supernatant was discarded and the pellet again suspended; this process was repeated for the four washes. After the bound radioactivity was eluted from the HAP with 0.5 ml of ethanol, the ethanol extract with 10 ml of scintillation fluid was counted for radioactivity in a Beckmann LS9000 liquid scintillation counter. Specific binding was calculated by subtracting nonspecific from total binding. The resulting data were analyzed by the method of Scatchard (1949) and the quantity of binding expressed as fmol/mg of cytosol protein, fmol/g of tissue, or fmol/mg DNA, according to the nature of the assay.

Steroid Specificity Determinations

The steroid specificity for the observed ligand binding to matrix AR_N was determined twice for the cancer specimens by using pooled samples of 5 specimens. Nuclear matrices were incubated with 2 nM of $[^3H]$R1881 plus a 1000-fold excess of TA with or without increasing concentrations of unlabelled competitors. Dexamethasone, progesterone, 17β-estradiol, DHT, and R1881 were used in excesses ranging from 2 to 1000-fold, as tabulated in Appendix 8, and the resultant binding of $[^3H]$R1881 was then measured.

Protein and DNA Assays

Protein concentrations were determined by the Bio•Rad Protein Assay (Bio•Rad, Richmond, California). The Bio•Rad protein assay procedure has been validated by comparison with the method of Lowry et al (1951) in this laboratory. DNA was measured in crude and purified nuclear preparations by the method of Burton (1956), using calf thymus DNA as the standard.

Light and Electron Microscopy

Purified nuclei and nuclear matrices stained with methylene blue were examined by light microscopy in some experiments using an Olympus EH in order to assess relative purity. Electron microscopy was performed in two laboratories. Initial samples were prepared for viewing in the laboratory of Dr. T. K. Shnitka, Department of Pathology, as follows: selected samples of purified prostate cancer nuclei and nuclear matrices were fixed at 4°C in 4% gluteraldehyde in 0.1 M cacodylate buffer (pH 7.3). The nuclei were then rinsed in two changes of cacodylate buffer and post-fixed in 1% OsO_4 (Caulfields' fixative). They were dehydrated through a graded ethanol series and then treated with propylene oxide. The nuclei were embedded in Epon 812 resin and sectioned on a Reichert OMU2 ultramicrotome. The sections were stained with uranyl acetate and lead citrate and photographed on a Siemens Elmiskop 1 electron microscope.

The electron micrographs reproduced below were obtained courtesy of the Muttart-Collip Memorial Electron Microscope Laboratory, and were prepared as described above, except that phosphate buffer was used rather than cacodylate buffer, LX-112 resin was used rather than Epon 812 resin, a Porter-Blum MT-2 ultramicrotome was used rather than a Reichert OMU2 ultramicrotome, and a Siemens Elmiskop 102 electron microscope was used rather than a Siemens Elmiskop 1 electron microscope.

Estrogen Exchange Assay

As described above, cytosols were prepared from BPH specimens, bound to HAP, and washed once with TM-PMSF buffer to remove any material not bound to HAP. Following 2-16 hours incubation in the presence of serial dilutions of 17β-estradiol, preparations were heated to 30°C in a water bath for 30 minutes with a control assay at 0°C, to determine whether the measured concentrations of ER might be increased by a heat-induced exchange of endogenous for exogenous estrogen. All incubations were performed in duplicate. Normal and BPH nuclear matrices from both ER_C-positive and ER_C-negative specimens were also washed and incubated as described for cytosols, with or without heating to 30°C for 30 minutes, to determine the presence or absence of extractable and matrix-bound ER_N, and to determine the role of the temperature exchange for the accurate quantification of extractable and matrix-bound ER_N.

Variations in Sucrose Sedimentation Requirements for Nuclear Purification for Benign Prostatic Hyperplasia

To determine the optimal concentration and volume of sucrose in nuclear sedimentation with respect to both nuclear purity and nuclear yield, nuclei from a series of BPH specimens were prepared as described above. However, a range of sucrose concentrations (1.8 M, 2.0 M, 2.2 M) were used, and also, the column heights were varied by using increasing volumes of the sucrose solutions (5 ml, 10 ml, and 15 ml of sucrose) for the nuclear sedimentation procedure. To estimate nuclear recovery, samples were taken for DNA assay from both the crude nuclear pellet (following preliminary centrifugation) and the post-sucrose sedimentation purified nuclear pellet. Percentage nuclear recovery was then calculated as the concentration of DNA in the purified nuclear pellet divided by the concentration of DNA in the crude nuclear pellet, expressed as a percentage. Nuclear purity was assessed by light microscopy (1000 x magnification) and the preparations were subjectively categorized as extremely pure, moderately pure, and impure. All preparations were prepared and assessed by the same investigator and interspecimen variation was minimized by performing assays on several specimens simultaneously. Light microscopy also provided an estimate of nuclear recovery for comparison with the DNA assays.

Variations in Methods of Pulverization and Homogenization Techniques for Nuclear Purification for Benign Prostatic Hyperplasia

To determine the optimal method of prostate tissue preparation for measurement of AR_N, a series of experiments was performed using BPH tissue, in which 1/3 of the specimen was prepared in the standard fashion (pulverization in liquid nitrogen, followed by homogenization on glass), 1/3 of the specimen was similarly pulverized but then homogenized using the Polytron PT-10 homogenizer (as described for the preparation of cytosol) and 1/3 of the specimen was chopped into fine pieces, not pulverized, and homogenized using the Polytron PT-10 homogenizer (see Figure 1). Specimens were then processed as described for the preparation of nuclear matrices, and assayed for AR_N in both extractable and matrix-bound fractions. Samples were taken for DNA assay from both crude and purified nuclei, in order to compare nuclear recoveries and to allow expression of results in fmol/mg nuclear DNA in addition to fmol/g of tissue.

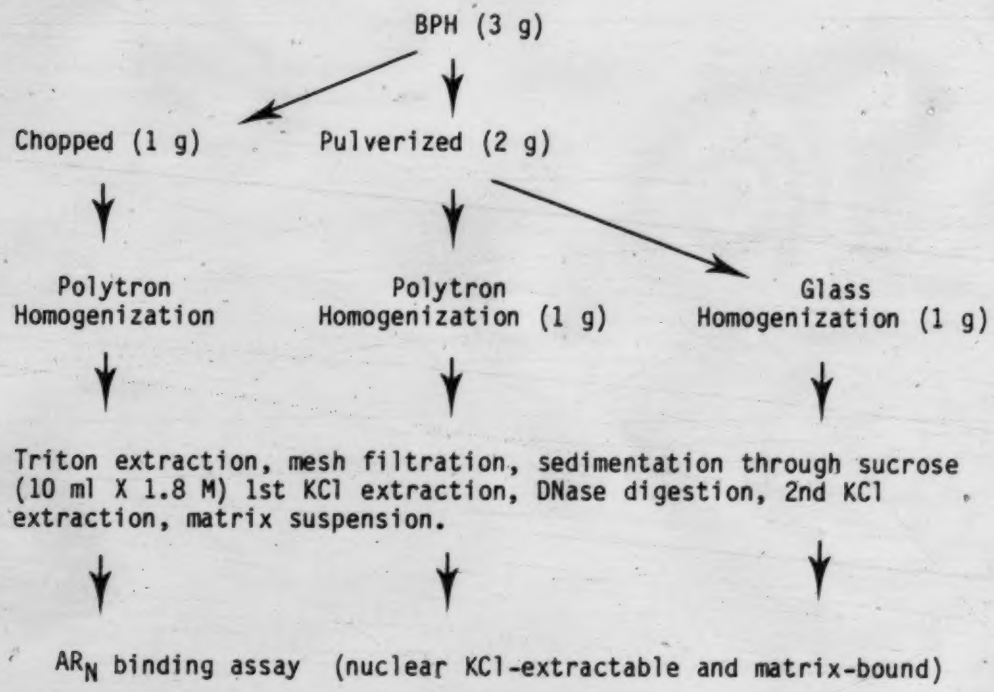
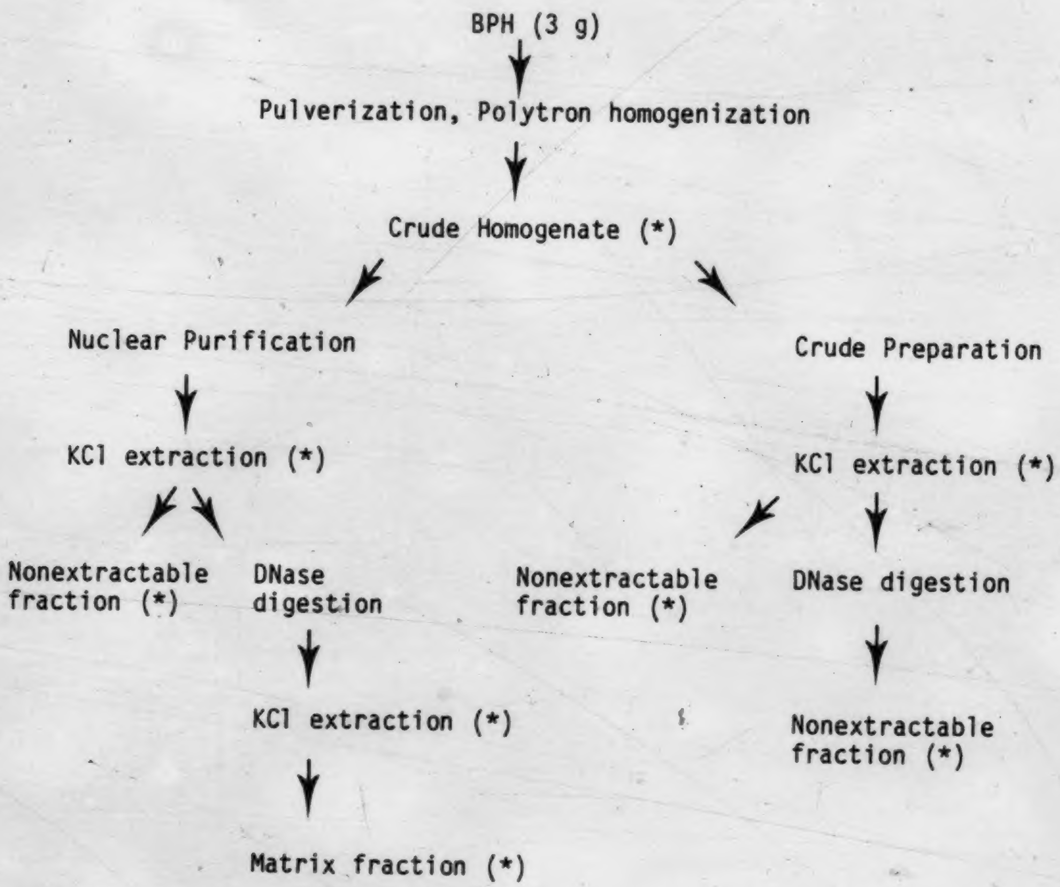

BPH (3 g)
Chopped (1 g)
Pulverized (2 g)
Polytron Homogenization
Polytron Homogenization (1 g)
Glass Homogenization (1 g)
Triton extraction, mesh filtration, sedimentation through sucrose (10 ml X 1.8 M) 1st KCl extraction, DNase digestion, 2nd KCl extraction, matrix suspension.
AR_N binding assay (nuclear KCl-extractable and matrix-bound)

Figure 1. Flow diagram for studies of variations in tissue pulverization and homogenization for nuclear purification. DNA assay was performed on samples from crude homogenate and post-sucrose purified nuclei, for each preparation.

Measurement of Androgen Receptor Concentration Following Different Extents of Nuclear Purification for Benign Prostatic Hyperplasia

To determine the optimal method of measurement of AR_N, a series of experiments was performed using BPH tissue (see Figure 2), in which approximately 3 g of BPH was first pulverized in liquid nitrogen, then homogenized using the Polytron PT-10 homogenizer, in 30 ml of STM-PMSF buffer. A portion (2 ml) of the crude homogenate was then directly bound to HAP and assayed for AR_N. The remaining homogenate was then divided into 2 equal portions of 14 ml, for separation into purified and crude preparations. The purified preparation was treated with Triton X-100, mesh filtration, and sucrose sedimentation as described above for the preparation of nuclear matrices, while the crude preparation was treated with Triton X-100 only. One half of each preparation was then extracted with KCl for 30 minutes, centrifuged at 10,000 x g for 10 minutes, to yield both extractable and non-extractable fractions. The remaining half of the purified preparation was treated with a 1st KCl extraction, DNase digestion and 2nd KCl extraction, to yield extractable and matrix-bound fractions. The remaining half of the crude preparation was treated with KCl extraction and DNase digestion to yield both extractable and non-extractable fractions. Samples were taken for DNA assay from the crude homogenate and the purified nuclear preparation, in order to allow calculation of nuclear recovery, and to allow expression of calculated binding in fmol/mg nuclear DNA in addition to fmol/g of tissue (see Figure 2).


BPH (3 g)
Pulverization, Polytron homogenization
Crude Homogenate (*)
Nuclear Purification
Crude Preparation
KCl extraction (*)
KCl extraction (*)
Nonextractable fraction (*)
DNase digestion
Nonextractable fraction (*)
DNase digestion
KCl extraction (*)
Nonextractable fraction (*)
Matrix fraction (*)

(*) denotes fractions assayed for AR_N.

Figure 2. Flow diagram for studies of AR_N measurement following different extents of nuclear purification.

CHAPTER III

RESULTS

Subcellular Concentrations of Androgen Receptor in Prostate Cancer

AR_C, AR_N-extractable, and AR_N-matrix concentrations were determined for 16 patients with prostate adenocarcinoma (see Table I and Appendix 4). AR_C was quantified in both fmol/mg cytosol protein and fmol/g of tissue. AR_N (1st extract, 2nd extract, and matrix-bound) was quantified in fmol/g of tissue. Although the protein concentration of the 1st KCl-extract was determined in all assays, thereby allowing expression of AR_N in fmol/mg of nuclear extract protein, we have not expressed our results in this fashion for two reasons. Primarily, the nature of this protein and its significance are undetermined, unlike cytosol protein, which has been repeatedly demonstrated to be a useful mode of expression for cytoplasmic receptor assays. Secondly, since the cytosol protein is unrelated to the extracted nuclear protein, it would be of no value to relate AR_C and AR_N concentrations when expressed as functions of their respective protein concentrations. In 8 of the 16 specimens, DNA assays were performed, thereby enabling expression of AR_N in fmol/mg of starting DNA (pre-purification), or fmol/mg of nuclear DNA (post-purification) the latter mode correcting for variable nuclear recovery. Alternatively, results expressed in fmol/g of tissue may be corrected for nuclear loss secondary to nuclear purification by dividing the result by the percent nuclear recovery to yield fmol/g of tissue (corrected) (see Table II).

The mean AR_C concentration, as shown in Table I and Figure 3, was 1028 fmol/g of tissue (range = 0-3938 fmol/g of tissue), with a mean K_d

TABLE I

Androgen Receptor in Adenocarcinoma of the Prostate

Preparation Assayed (n=16)	Androgen Receptor Concentration				Kd§ (nM)	Number of positive samples	Percent of total AR†	Percent of AR_N‡
	fmol § / mg Protein	fmol § / g of tissue	fmol*§ / mg starting DNA	fmol*§ / mg nuclear DNA				
Cytoplasm	55 (±20)	1028 (±255)	-	-	0.3 (±0.1)	14	61	-
First Extract**	-	140 (± 26)	48 (± 9)	87 (± 17)	1.1 (±0.4)	12	8	20
Second Extract	-	111 (± 37)	17 (± 7)	26 (± 10)	1.7 (±1.2)	3	6	16
Total Extract	-	251 (± 31)	65 (± 8)	113 (± 15)	-	12	14	36
Matrix-bound	-	396 (±112)	148 (± 50)	336 (±128)	0.8 (±0.1)	14	25	64
Total Nuclear	-	547 (±128)	213 (± 48)	449 (±121)	-	15	39	100
Total Cellular	-	1675 (±295)	-	-	-	15	100	-

§ mean of AR-positive samples only (± S.E.M.); AR-positive = receptor concentration > 10 fmol/g of tissue

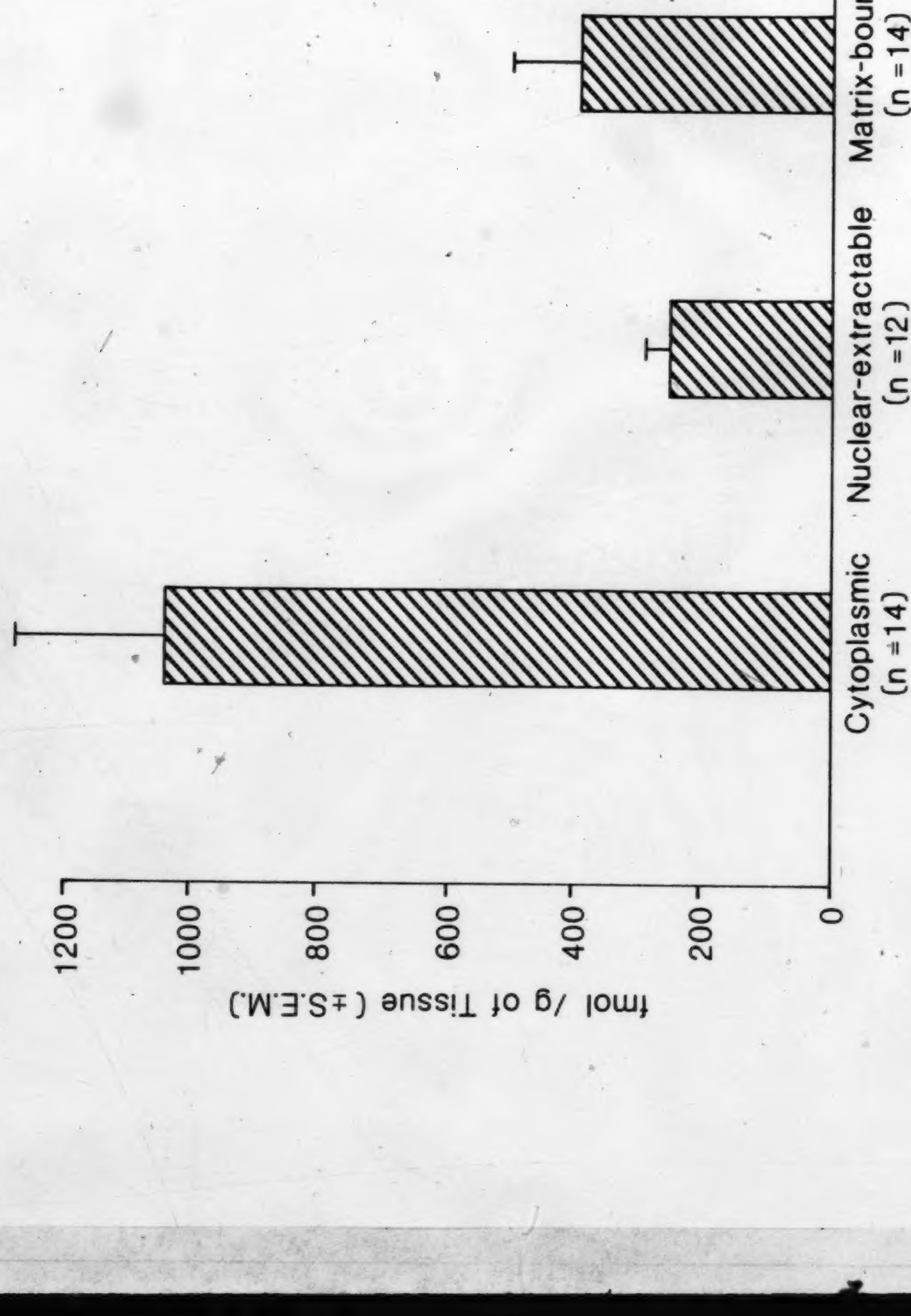
* n=8

† percent of total cellular content of AR (AR_C + AR_N) when calculated in fmol/g of tissue

‡ percent of total AR_N when calculated in fmol/g of tissue

** nuclear AR extracted by 0.6 M KCl (see Materials and Methods)

Figure 3. Androgen receptor of prostate cancer. Bars represent mean AR concentration in fmol/g of tissue ± S.E.M. (n=16), and percentage of total cellular receptor, for AR-positive samples, using HAP assay, R1881, and Scatchard analyses (n = number of positive samples of the 16 assayed specimens). AR-positive is defined as specific binding > 10 fmol/g of tissue.


fmol /g of Tissue (±S.E.M.)
0
200
400
600
800
1000
1200
Percentage of Total Cellular Receptor
0
10
20
30
40
50
60
70
80
Cytoplasmic (n =14)
Nuclear-extractable (n =12)
Matrix-bound (n =14)

of 0.3 nM (range = 0.1-1.1 nM) (see Table I and Figure 3). Both patients who had no detectable AR_C had received radiotherapy previously (#4, #5 of Appendix 4), and one of these also had no detectable AR_N (#4). However, the third radiotherapy patient had detectable AR_C and AR_N (#16).

The first KCl extract contains AR_N which is relatively easily extracted from the nucleus, while the second KCl extract contains AR_N which is extractable only after DNase I digestion (Donnelly, 1982). The mean AR_N concentration in the first KCl extract of AR-positive specimens (AR > 10 fmol/g of tissue) was 140 fmol/g of tissue (range for all 16 specimens = 0-253 fmol/g of tissue), with a mean K_d of 1.1 nM (range = 0.2-4.4 nM). The mean AR_N concentration in the second KCl extract of AR-positive specimens was 111 fmol/g of tissue (range for all 16 specimens = 0-184 fmol/g of tissue), with a mean K_d of 1.7 nM (range = 0.3-4.2 nM). The sum of AR_N in the first and second extracts therefore represents the total extractable AR_N (251 fmol/g of tissue, range = 0-341 fmol/g of tissue). However, since 12/16 specimens contained AR_N in the first extract, and only 3/16 specimens contained AR_N in the second extract, the majority of salt-extractable AR_N is contained in the first extract. It is noteworthy that of the 4/16 specimens which contained no detectable extractable AR_N, only one also had no detectable matrix-bound AR_N (#4 of Appendix 4), while 3 had significant quantities of matrix-bound AR_N (#3, 6, 10). The significance of the finding of extractable AR_N in the absence of matrix AR_N in one patient (#7) is as yet undetermined, but it is noteworthy that this patient died of metastatic disease three months after initiation of hormonal therapy.

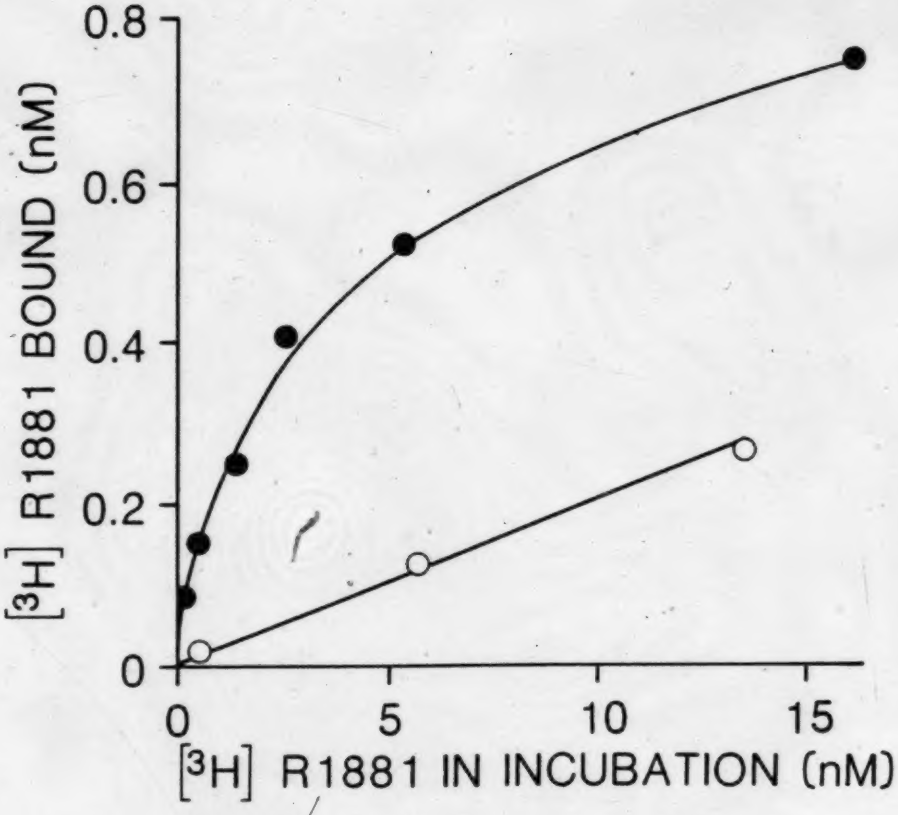
The mean matrix-bound AR_N concentration of AR-positive specimens was 396 fmol/g of tissue (range for all 16 specimens = 0-1330 fmol/g of tissue), with a mean K_d of 0.8 nM (range = 0.3-1.7 nM). A representative saturation analysis and derived Scatchard analysis from the same specimen (#15 of Appendix 4) are depicted in Figures 4 and 5 respectively. Although one of the two specimens with no detectable matrix-bound AR_N had detectable extractable AR_N (#7) as noted above, the other patient had no detectable AR in any other cellular compartment (#4). Table I also provides mean AR_N concentrations expressed as fmol per mg of starting DNA, and per mg of nuclear DNA. The histogram display of the data in Figure 3 illustrates both the greater concentration and variability of AR_C as compared to AR_N.

Linear regression analysis revealed no correlation between Gleason score of the tumour and the concentration of the various components of AR_N, whether expressed as fmol/g of tissue or fmol/mg of nuclear DNA. There was a marginally positive correlation ($r = 0.60$) between extractable and matrix-bound AR_N, but only when expressed as fmol/mg nuclear DNA. As expected, there was no correlation between AR_C and extractable, matrix-bound, or total AR_N (data not shown).

Studies of DNA Concentration in Prostate Cancer

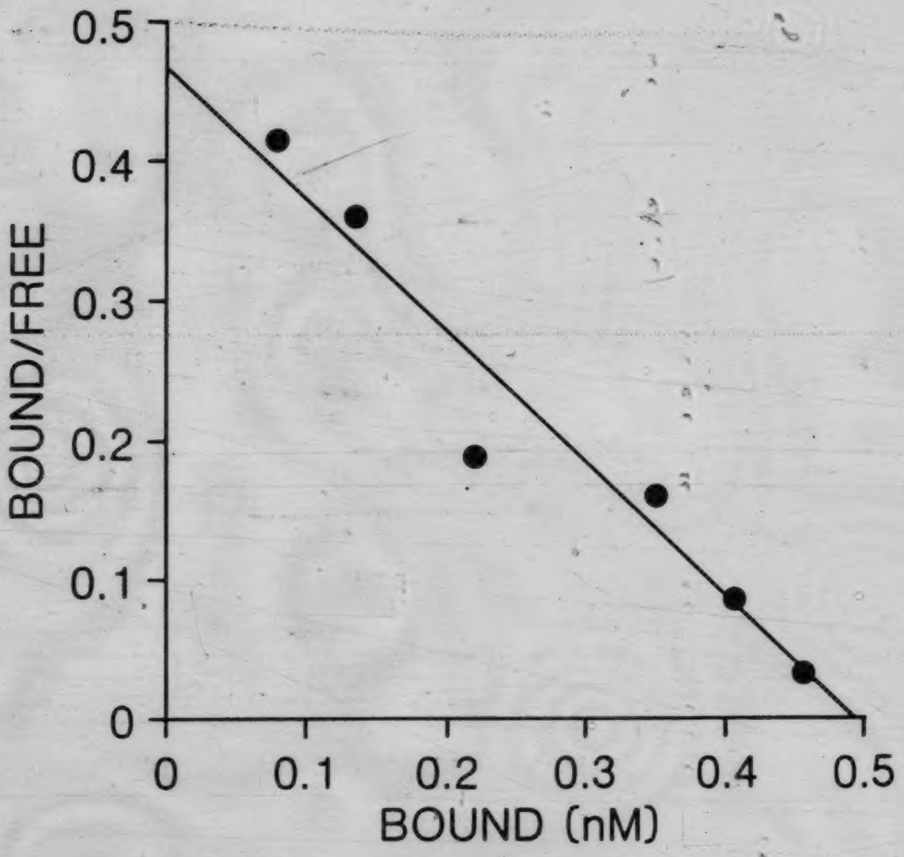
In order to determine the optimal means of expression of AR_N concentration, it was necessary to first measure the DNA concentration of the specimens. From this data, expressed as mg of DNA/g of tissue, binding could then be expressed as fmol/mg of DNA, in addition to fmol/g of tissue. If the receptor assay was performed on a crude pellet, it would be reasonable to assume that the amount of DNA in the initial homogenate would correlate with the number of nuclei present, and

Figure 4. Saturation analysis for [^{3}H]R1881 binding to nuclear matrix of adenocarcinoma of the prostate. Total binding (●). Non-specific binding (○).



ADENOCARCINOMA OF PROSTATE
SATURATION ANALYSIS - AR_N (MATRIX)

Figure 5. Scatchard analysis for [^{3}H]R1881 binding to nuclear matrix of adenocarcinoma of the prostate. Maximum binding = 1281 fmol/g of tissue; $r = 0.97$; $K_d = 1.0$ nM.


0.5
0.4
0.3
0.2
0.1
0
BOUND/FREE
0
0.1
0.2
0.3
0.4
0.5
BOUND (nM)

4

therefore with the amount of AR_N within those nuclei. However, because of the inevitable loss of nuclei during nuclear purification, any receptor assay performed on a purified nuclear preparation must be accompanied by determination of both starting DNA concentration (DNA concentration of the crude homogenate), and nuclear DNA concentration (DNA concentration following nuclear purification). Comparison of the starting and nuclear DNA concentrations allows estimation of nuclear recovery.

In the 8 cancer specimens in which DNA concentrations were measured, the mean nuclear recovery was 55 percent (range = 26-76 percent) (see Table II). Results can therefore be expressed as fmol/mg starting DNA, fmol/mg nuclear DNA, or fmol/g of tissue, corrected for loss of nuclei, allowing expression as fmol/g of tissue (corrected). Table II provides comparison of these parameters for matrix-bound AR_N. It is readily observed that from the basic mode of expression (fmol/g of tissue), a wide range of binding values is created by introducing the variables of mg of homogenate DNA/g of tissue (starting DNA concentration) or mg of nuclear DNA/g of tissue (nuclear DNA concentration). Since nuclear recovery averaged 55 percent, the mg of nuclear DNA/g of tissue is less than that observed for the homogenate. Therefore, binding expressed as fmol/mg nuclear DNA is approximately twice that of the corresponding value obtained using the DNA concentration of the initial homogenate.

Further variability is introduced by the wide range of nuclear recovery seen in our samples (for example, #14 has a 3.8-fold increase in fmol/mg DNA when corrected for nuclear recovery, while the same correction produces only 1.4-fold increase for #15). The mean DNA

TABLE II

Variations in Expression of Matrix-bound AR_N

Patient #	DNA Concentration Homogenate (mg/g)	DNA Concentration Nuclei (mg/g)	Nuclear Recovery (%)	AR Concentration $\frac{\text{fmol}}{\text{mg starting DNA}}$ *	AR Concentration $\frac{\text{fmol}}{\text{mg nuclear DNA}}$ **	AR Concentration $\frac{\text{fmol}}{\text{g of tissue (corrected)}}$ ***
3	15.90	7.65	48	14	30	487
4	0.21	0.16	76	0	0	0
5	5.94	3.65	61	16	26	157
7	0.72	0.43	60	0	0	0
9	9.92	5.50	55	134	242	2418
12	4.20	1.88	45	167	373	1595
14	1.97	0.52	26	228	860	1727
15	3.87	2.64	68	331	485	1883
Mean †	5.34	2.80	55	148	336	1378

* $\frac{\text{fmol}}{\text{g of tissue}} \div \frac{\text{mg homogenate DNA}}{\text{g of tissue}}$

** $\frac{\text{fmol}}{\text{g of tissue}} \div \frac{\text{mg nuclear DNA}}{\text{g of tissue}}$

*** $\frac{\text{fmol}}{\text{g of tissue}} \div$ percent nuclear recovery X 100

† Mean calculated for AR-positive samples only; AR-positive = receptor concentration > 10 fmol/g of tissue

concentration was 5.34 mg/g of tissue in the homogenate (range = 0.21-15.90 mg/g of tissue) and 2.80 mg/g of tissue following nuclear purification (range = 0.16 - 7.65 mg/g of tissue). The mean matrix-bound AR_N concentration was 148 fmol/mg starting DNA (range = 0-331 fmol/mg starting DNA), 336 fmol/mg nuclear DNA (range = 0-485 fmol/mg nuclear DNA), and 1378 fmol/g of tissue (corrected) (range = 0-2418 fmol/g of tissue (corrected)). Corresponding AR_N concentrations for the first, second, and total extractable AR_N, plus total nuclear AR_N, are provided in Appendix 4, with corresponding mean values provided in Table I.

Cytoplasmic Progesterone Receptor in Prostate Cancer

The concentrations of PgR_C in all 16 specimens of prostatic adenocarcinoma are shown in Table III and Appendix 5. PgR_C was detectable in 11 of the 16 specimens, with a mean concentration of 945 fmol/g of tissue, or 44 fmol/mg of cytosol protein (range = 0-3253 fmol/g of tissue, or 0-165 fmol/mg of cytosol protein), and a mean K_d of 0.9 nM (range = 0.1-3.9 nM) (see Table III). There was no obvious correlation between absence of PgR_C and absence of other cytoplasmic receptors, in that absence of PgR_C occurred with presence of AR_C and ER_C (n=1), absence of AR_C and ER_C (n=1), and presence of AR_C with absence of ER_C (n=3) (see Appendix 5).

Of the 5 patients with no measureable PgR_C, 2 had disease progression or death, and 3 had disease stabilization or regression. The mean PgR_C in those with progression or death was 324 ± 110 fmol/g of tissue (± S.E.M.), while the mean PgR_C in those with regression or stabilization was 903 ± 339 fmol/g of tissue (± S.E.M.). The observed

TABLE III

Cytoplasmic Progesterone and Estrogen Receptor in Adenocarcinoma of the Prostate

Receptor Assayed	Proportion of Receptor-Positive Samples	Receptor Concentration		Kd (nM)*
		fmol/g of tissue*	fmol/mg of cytosol protein*	
PgR_C	11/16	945 ± 253	44 ± 14	0.9 ± 0.3
ER_C	6/16	278 ± 41	13 ± 4	0.4 ± 0.1

* Mean ± S.E.M. for receptor-positive samples (receptor concentration > 3 fmol/mg of cytosol protein); n=16.

difference is not statistically significant using the Student's t-test ($p > 0.05$) however, due to the extreme variation in binding in the two patient groups.

Cytoplasmic Estrogen Receptor in Prostate Cancer

The concentrations of ER_C in all 16 specimens of prostatic adenocarcinoma are shown in Table III and Appendix 5. ER_C was detectable in 6 of the 16 specimens, with a mean concentration of 278 fmol/g of tissue, or 13 fmol/mg of cytosol protein (range = 0-387 fmol/g of tissue, or 0-34 fmol/mg of cytosol protein), and a mean K_d of 0.4 nM (range = 0.1-1.0 nM) (see Table III).

Of the 6 patients with measureable ER_C, 3 had disease progression or death, and 3 had disease regression or stabilization. Similarly, of the 10 patients with no measureable ER_C, 5 had disease progression or death, and 5 had disease regression or stabilization. This parameter was therefore not a useful prognostic index for adenocarcinoma of the prostate in this series of patients.

Course of Disease and Androgen Receptor Concentration - Correlations for Prostate Cancer Patients

According to the National Prostatic Cancer Project Response Criteria (see Appendix 1), the 16 patients were initially categorized as objective complete response (n=1), objective partial regression (n=3), objective stable (n=5), objective progression (n=3), or death (n=4) (see Appendix 3). Because of the small number in each group, similar outcome groups were combined to form the categories of objective regression or stabilization (n=9), and objective progression or death (n=7).

TABLE IV

Androgen Receptor Concentration and Disease Response for Adenocarcinoma of the Prostate

Receptor Assayed	Death/Progression (n=7)		Stable/Regression (n=9)	
	AR Concentration*	Proportion** AR-positive	AR Concentration*	Proportion** AR-positive
AR_C	575 ± 210 (670 ± 232)	6/7	1152 ± 379 (1296 ± 398)	8/9
AR_N (extractable)	39 ± 15 (68 ± 15)	4/7	178 ± 38 (200 ± 35)	8/9
AR_N (matrix-bound)	102 ± 33 (143 ± 29)	5/7	535 ± 161 (535 ± 161)	9/9

* Mean values expressed in fmol/g of tissue ± S.E.M. Upper figures represent calculation based on total sample. Lower figures in parentheses represent calculation based only on positive samples (AR positive).

** AR-positive = receptor concentration > 10 fmol/g of tissue.

Figure 6. Androgen receptor and disease response for adenocarcinoma of the prostate. Open bars represent mean AR concentrations in cytoplasmic, salt-extractable, and matrix-bound fractions (fmol/g of tissue ± S.E.M.) for patients with objective evidence of disease progression or death (n=7). Shaded bars represent mean AR concentrations for patients with objective evidence of disease stabilization or regression (n=9).

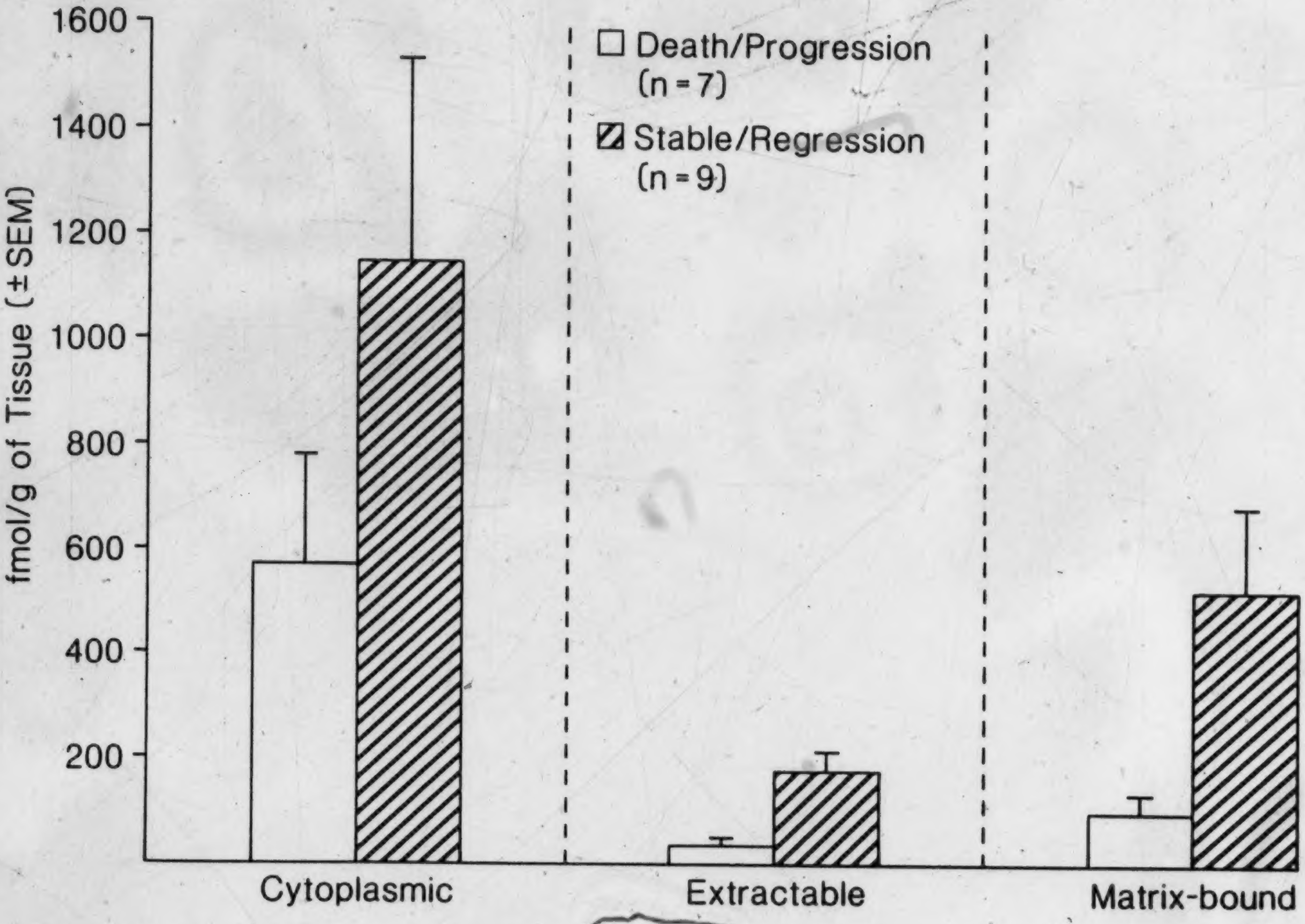

1600
1400
1200
1000
800
600
400
200
fmol/g of Tissue (± SEM)
☐ Death/Progression
(n = 7)
▨ Stable/Regression
(n = 9)
Cytoplasmic
Extractable
Matrix-bound

Table IV outlines the relevant AR data of these two groups, and Figure 6 is a graphic representation of the same information. The mean values and S.E.M. presented in Table IV and Figure 6 are calculated using all assay values, including those in which there was no detectable binding, since the objective was to compare receptor values in all patients with progression or regression of disease. If only those patients with measureable receptor are examined, the mean values and S.E.M. are as depicted in Table IV by the figures in parentheses.

Statistical comparisons between the various receptor concentrations and their ability to predict disease outcome are listed in Table V. AR_C concentration did not correlate with disease response, since there was no significant difference between the AR_C concentration for those patients with disease regression or stabilization and those with disease progression or death, as analyzed by the Student's t-test. Conversely, both extractable and matrix-bound AR_N concentrations did correlate with disease response, whether all specimens or only those with measureable AR_N were included in the analysis. The coefficient of variation (see Table V) is a measure of relative variation about the mean between two samples, corrected for differences in the magnitude of individual values between the samples. There are minor differences in the coefficient of variation between extractable and matrix-bound AR_N, but both are considerably less than that observed for AR_C. The highest level of significance ($p < 0.02$) was seen with comparison of total AR_N concentrations between the two patient groups. In summary, both extractable and matrix-bound AR_N concentrations correlate with disease response, but the best correlation is obtained with total AR_N concentration.

TABLE V

Statistical Evaluation of Androgen Receptor Concentration
and Prediction of Disease Response in Adenocarcinoma of the Prostate

Receptor Analyzed	Total Sample		Positive Values Only	
	t-test *	Coefficient of Variation †	t-test *	Coefficient of Variation †
AR_C	NS §	1.62	NS §	1.49
AR_N (extractable)	$p<0.001$	0.64	$p<0.05$	0.63
AR_N (matrix-bound)	$p<0.05$	0.76	$p<0.05$	0.76
AR_N (total)	$p<0.02$	0.68	$p<0.02$	0.69

* Student's two sample t-test for unpaired variables (regression or stabilization versus progression or death)

† Coefficient of variation = $\frac{S\,(\overline{x} - \overline{y})}{\overline{x} - \overline{y}}$ where S = standard deviation

§ NS = Not significant ($p>0.05$)

Steroid Specificity for Binding of [^{3}H]R1881 to the Nuclear Matrix in Prostate Cancer

In order to determine whether the observed binding of [^{3}H]R1881 to nuclear matrices was to sites specific for androgenic steroids, steroid competition studies were performed on 2 pooled samples of prostate cancer matrices, each derived from 5 cancer specimens. The results for the 2 samples were very similar, and one is depicted in Figure 7. Both R1881 and DHT inhibit [^{3}H]R1881 binding to the nuclear matrix. Progesterone is slightly inhibitory at high concentrations, as has been previously reported for other prostatic AR (Hicks and Walsh, 1979; Donnelly, 1982), while dexamethasone and estradiol do not inhibit R1881 binding to the matrix sites.

Electron Microscopy of Purified Nuclei and Nuclear Matrices of Prostate Cancer

In order to be certain that our methods of nuclear purification and matrix preparation were as adequate for prostate cancer specimens as had been previously demonstrated for benign specimens by Donnelly (1982), it was necessary to obtain electron microscopic confirmation of nuclear purification and matrix preservation. Figure 8 is an electron micrograph of an isolated prostate cancer cell nucleus (x 15,000 magnification). Figure 9 (x 16,000 magnification) and Figure 10 (x 18,000 magnification) are electron micrographs of isolated prostate cancer nuclear matrices. Figure 11 is an electron micrograph of a group of prostate cancer nuclear matrices (x 15,000 magnification).

Although the structures of interest are surrounded by cell debris in these photomicrographs, these preparations are much purer than crude

Figure 7. Steroid specificity for [^{3}H]R1881 binding to nuclear matrix in adenocarcinoma of the prostate. Nuclear matrix preparation from pooled cancer specimens was adsorbed to HAP for 1 hour and incubated with 2 nM of [^{3}H]R1881 plus a 1000-fold excess of TA with or without increasing concentrations of the indicated unlabelled steroids. The HAP pellets were washed with phosphate buffer and the residual radioactivity determined. Total binding (100%) and binding in the presence of unlabelled R1881 (◆), dihydrotestosterone (△), progesterone (▲), estradiol (○), and dexamethasone (●) are depicted.

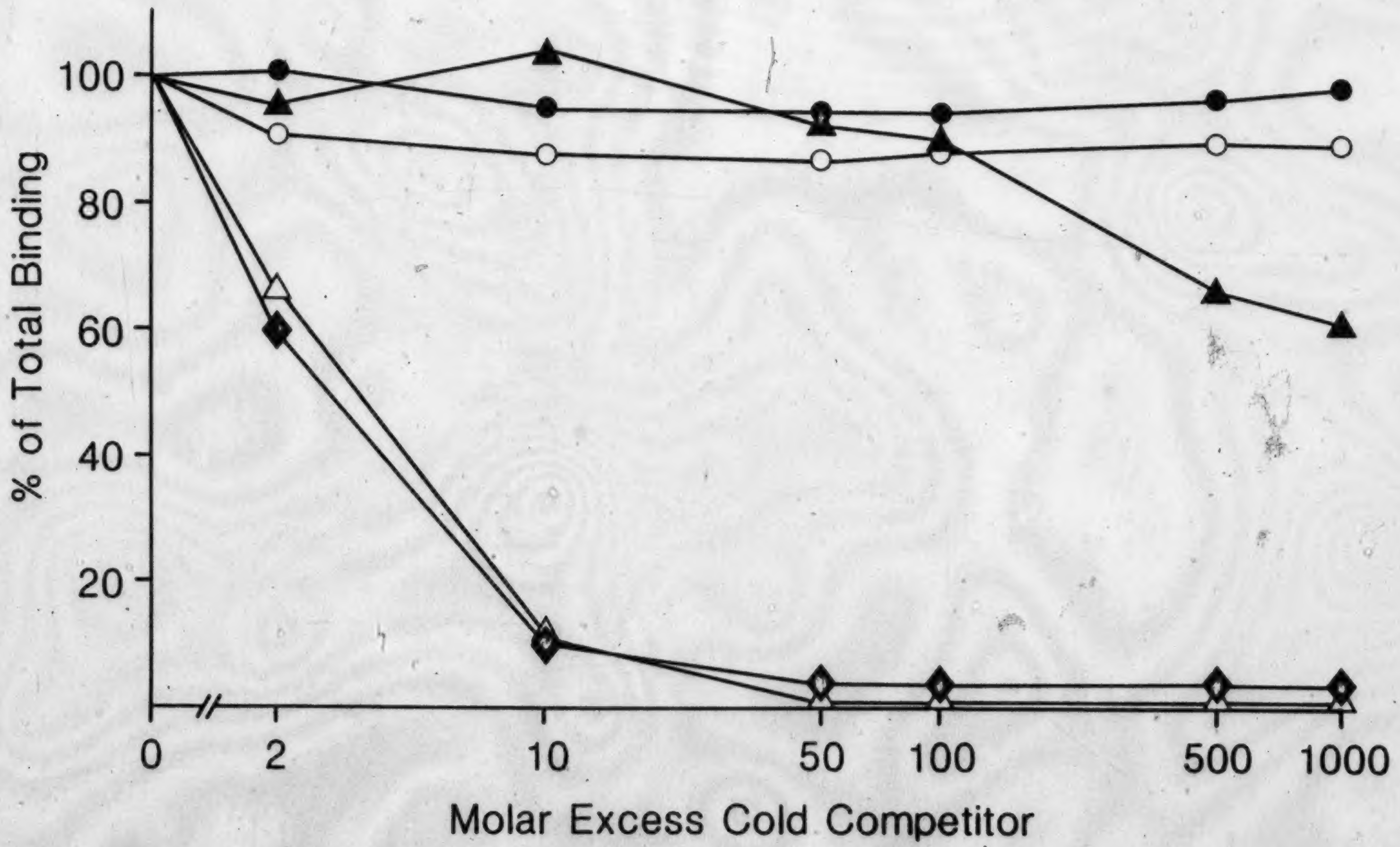

100
80
60
40
20
% of Total Binding
0
2
10
50
100
500
1000
Molar Excess Cold Competitor

Figure 8. Electron micrograph of prostate cancer cell nucleus (x 15,000 magnification).

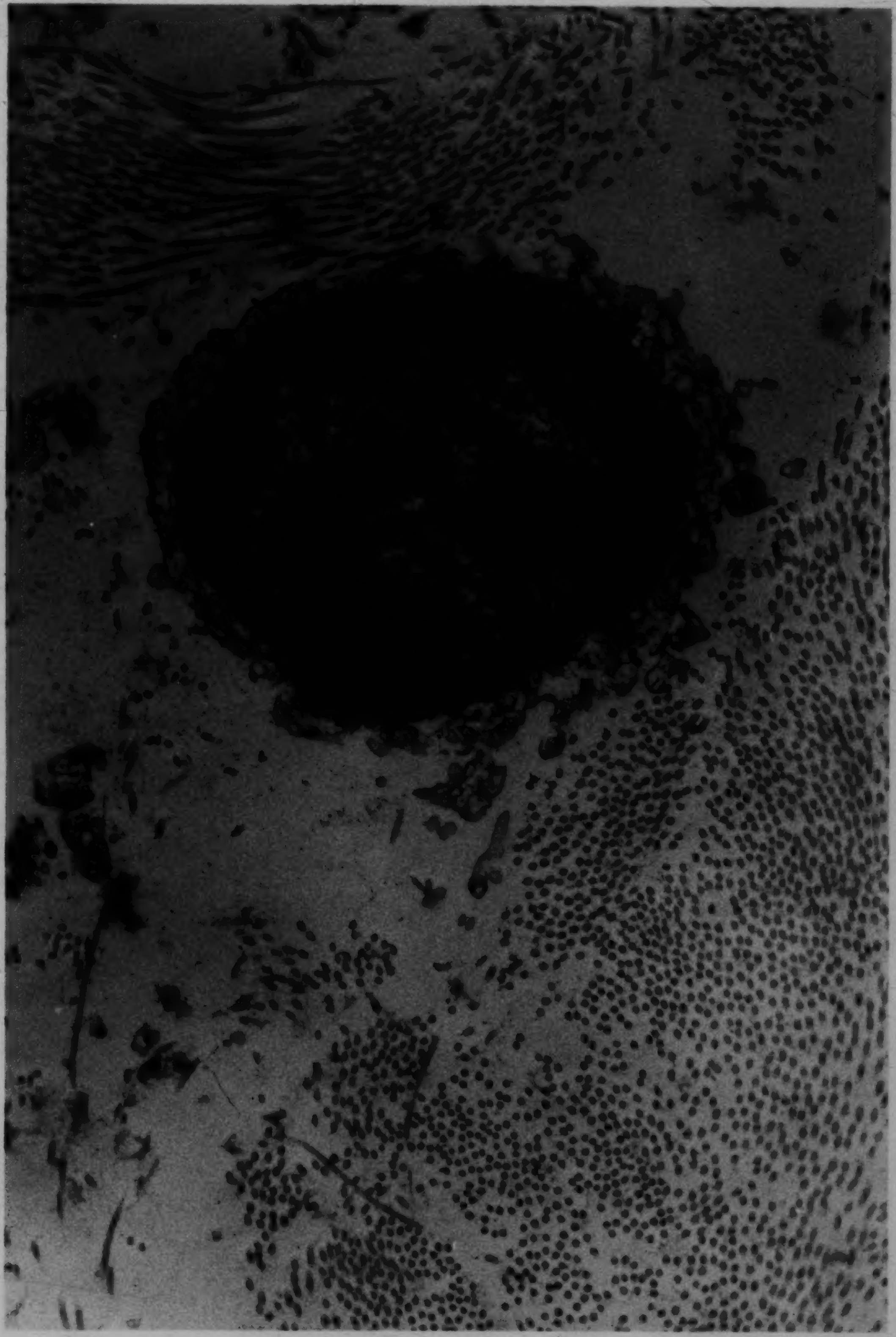


Figure 9. Electron micrograph of prostate cancer nuclear matrix (x 16,000 magnification).

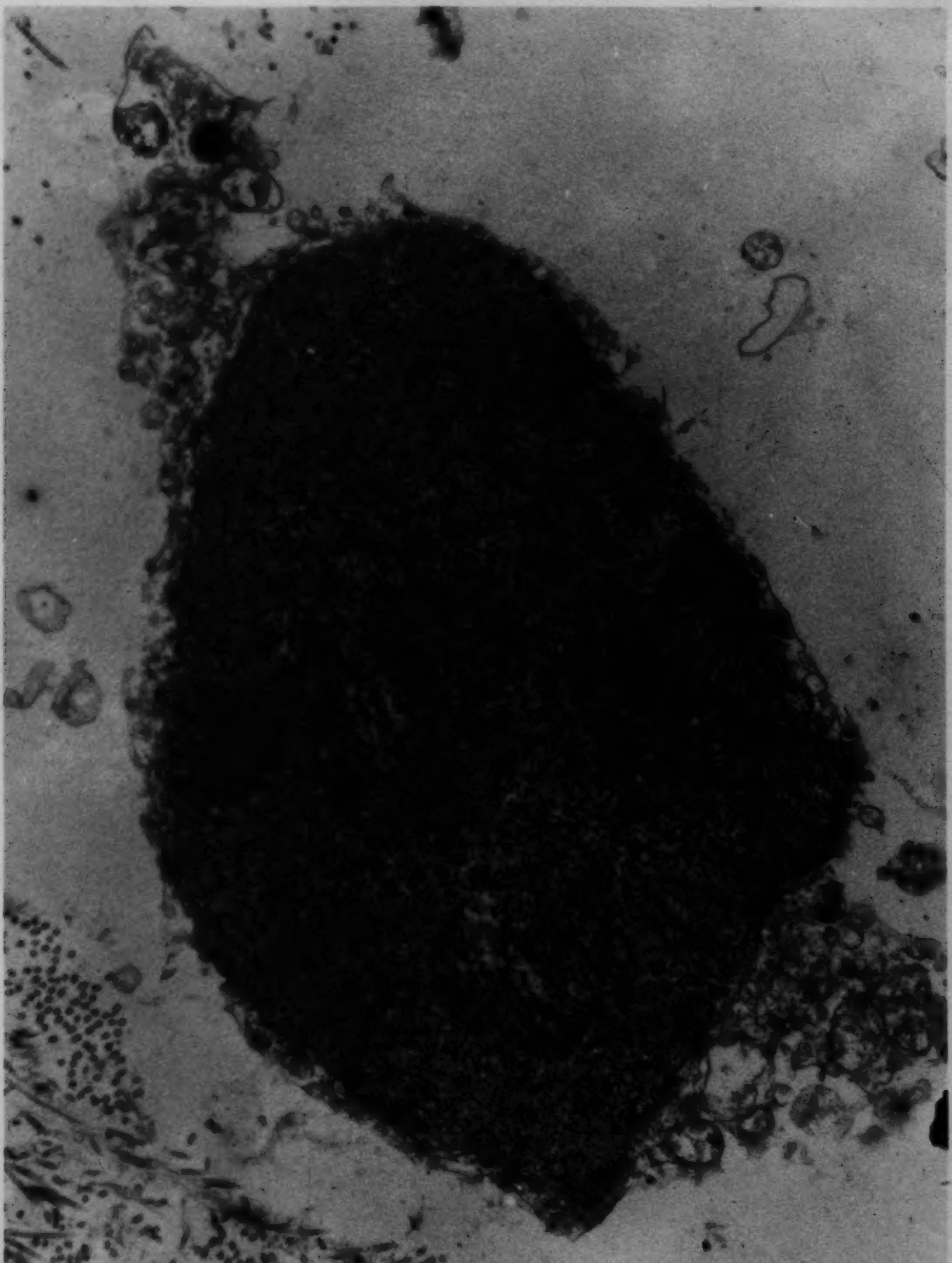


Figure 10. Electron micrograph or prostate cancer nuclear matrix (x 18,000 magnification).

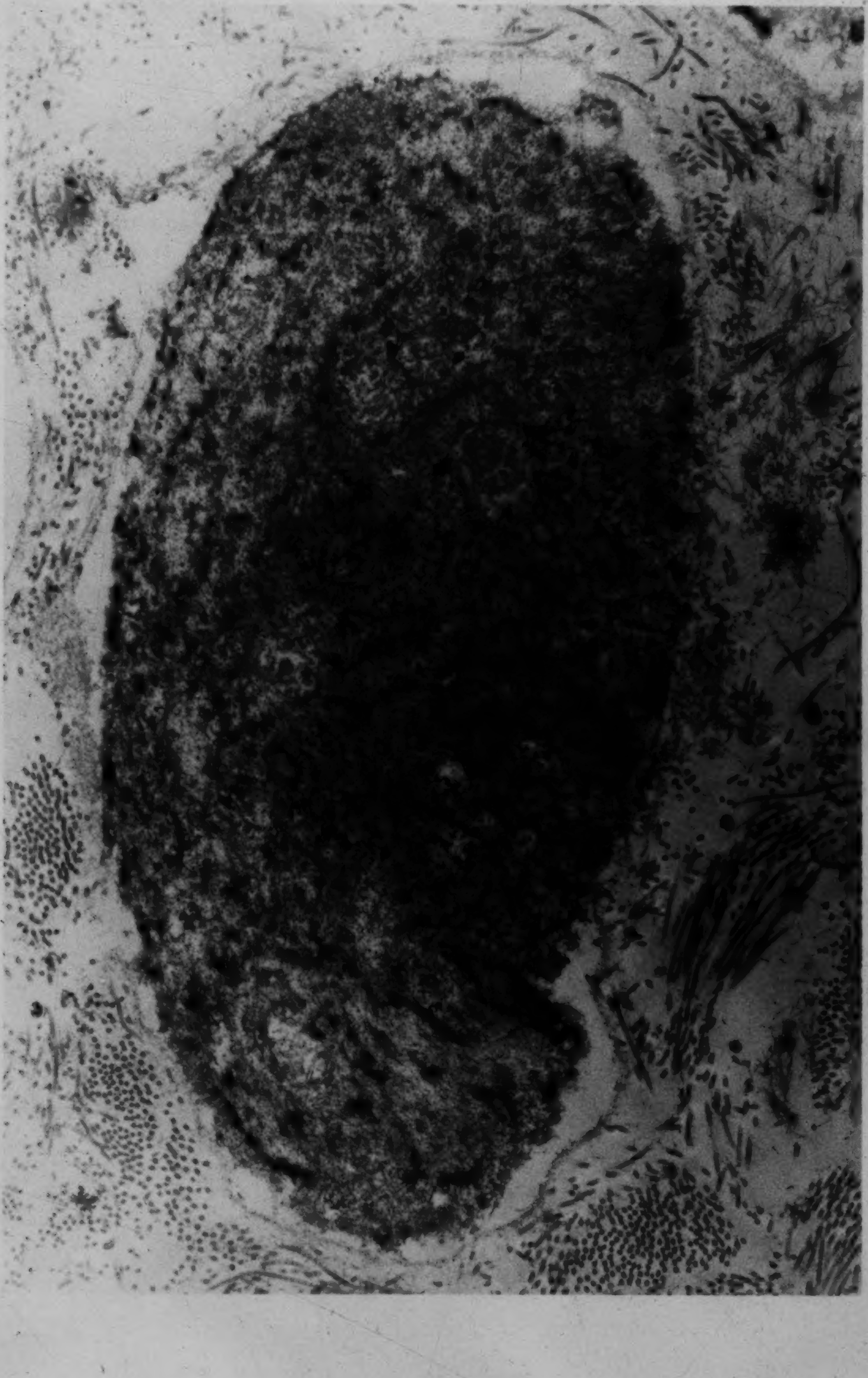
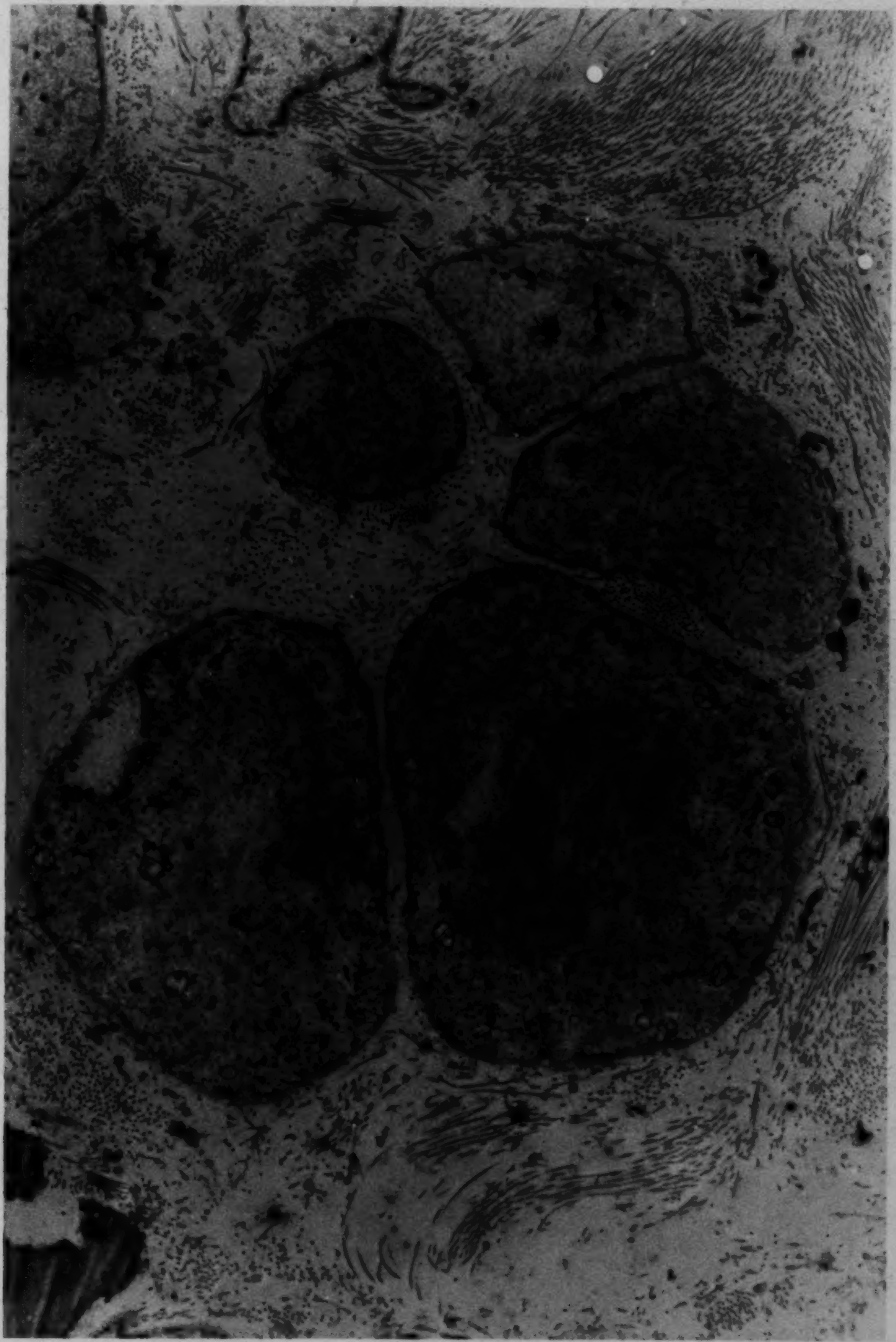


Figure 11. Electron micrograph of prostate cancer nuclear matrices (x 15,000 magnification).

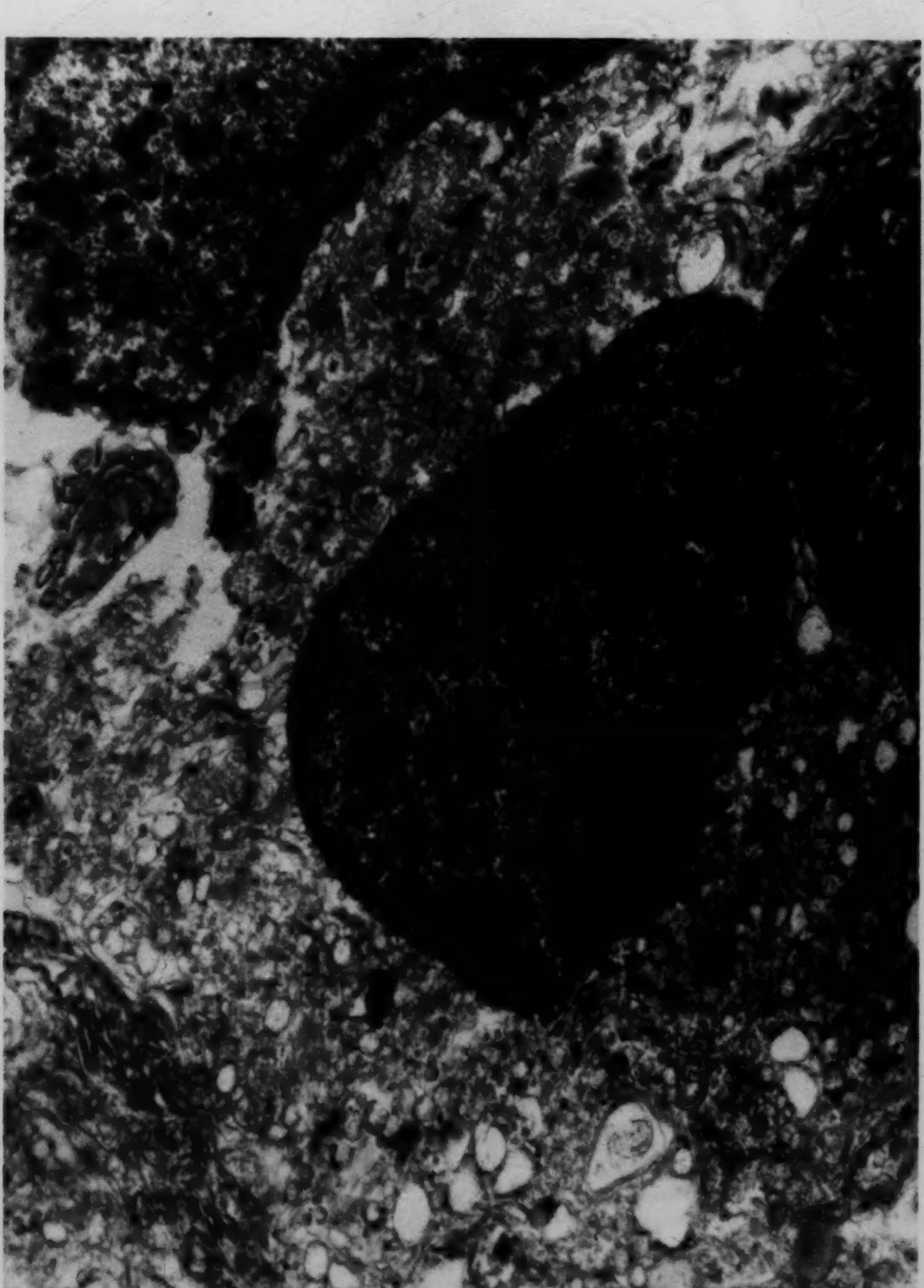


preparations of prostate cancer specimens, as documented by Figure 12 (x 18,000 magnification). These photomicrographs therefore demonstrate the relative purity of our nuclear preparations, and adequate nuclear matrix preservation.

Studies of Type I and Type II Estrogen Receptors in Rat Uteri

In order to provide a model for the investigation of multiple estrogen binding sites in the human prostate, a series of preliminary experiments were performed using the rat uterus, to quantify type I and type II cytoplasmic estrogen binding sites. Previous experiments in our laboratory had suggested that type II ER_C might be revealed in the presence of the sulfhydryl oxidizing agent sodium tetrathionate (NaTT) in rats injected *in vivo* with estradiol (data not shown). For the first experiments reported here, animals were injected with estradiol, but no ER_C could be detected, both in the absence and presence of NaTT (data not shown). Since it was possible that the Tris-EDTA-Glycerol (TEG, see below) buffer used for these experiments was inactivating type II sites, an experiment was performed using a single saturating dose of 10 nM 17β-estradiol, measuring ER_C with a variety of different buffers including STM, STM-glycerol (STM plus 10 per cent (v/v) glycerol), TE (10 mM Tris, 1.5 mM EDTA), TEG (10 mM Tris, 1.5 mM EDTA, 10 per cent (v/v) glycerol), TE-sucrose (10 mM Tris, 1.5 mM EDTA, 0.25 M sucrose), again in the presence or absence of NaTT. The results (not shown) again showed no demonstrable ER_C. A similar experiment using 0, 1 and 2 mM NaTT in the presence or absence of glycerol again revealed no ER_C binding. These preliminary experiments (data not shown) suggested that after estradiol injection *in vivo*, very little ER remains in the cytoplasm of the rat

Figure 12. Electron micrograph of prostate cancer crude nuclear preparation (x 18,000 magnification).



uterus, and that ER_C is not demonstrable in this situation, even in the presence of NaTT. The reason for the earlier observation that NaTT could be used to reveal estrogen binding in the uterine cytosol of estradiol-treated rats is as yet unresolved.

Attention was then directed to the unstimulated immature rat uterus, presumably in which most ER would not be translocated into the nucleus, as ovarian production of estradiol would be minimal. Scatchard analyses using 12 point Scatchard plots with an expanded upper range of steroid concentrations (see Appendix 7) to reveal binding to low affinity sites (such as ER type II sites) did not reveal type II ER_C binding, although type I ER_C was consistently present (data not shown). This experiment was repeated with the following modifications (data not shown):

a) comparison of ER_C in cytosols to which ligand was added prior to HAP binding as opposed to addition of ligand following HAP binding.

b) comparison of ER_C using DCC and HAP assays.

c) elimination of rotary shaking during HAP binding, to avoid any possibility of heat-induced inactivation of type II ER_C by the mechanical shaker.

d) use of a 30°C for 30 minute heat exchange to reveal endogenously filled ER_C.

e) comparison of ER_C in frozen uteri with frozen cytosol.

f) variable time of incubation with radioligand, ranging from 2-20 hours.

g) addition of the known receptor stabilizer sodium molybdate to cytsol buffers.

Despite these rather comprehensive attempts, it was not possible to clearly or consistently demonstrate the presence of type II ER_C in the immature rat uterine cytosol; more work will be required to duplicate the findings of Clark's group (Eriksson et al, 1978) for both type I and type II estrogen binding before this concept can be extended to the human prostate.

Estrogen Receptor in Benign Prostatic Hyperplasia

ER_C concentrations were determined in a series of 6 BPH specimens of which three were ER_C-negative (ER concentration < 3 fmol/mg of cytosol protein), and 3 were ER_C-positive (mean ER_C concentration = 8 fmol/mg of cytosol protein, mean K_d = 0.7 nM). Duplicate incubations subjected to 30°C heating for 30 minutes did not show any change in measured ER_C concentration in any of the specimens (data not shown).

ER_N (extractable and matrix-bound) was not detectable in 2 normal prostate specimens, and 9 BPH specimens. In 3 of the BPH specimens, a 30°C, 30 minute temperature exchange also revealed no detectable ER_N. In order to avoid the possibility of receptor denaturation during storage at -70°C for long periods, ER_N assays were performed on 2 additional BPH specimens, within 24 hours of obtaining the tissue. No detectable ER_N was present in either of these fresh specimens, with or without a 30°C, 30 minute temperature exchange (data not shown).

Studies of Variations in Methods of Nuclear Purification for Benign Prostatic Hyperplasia

In order to achieve optimal nuclear purification while maintaining a reasonable nuclear yield, it was decided to determine both nuclear purity (by light microscopy), and nuclear yield (by DNA assay) in BPH specimens which had been sedimented through variable sucrose concentrations and column heights. The results of these studies are shown in Table VI. All experiments were performed on 2 different BPH samples, and all numerical values are the means of the 2 results so obtained. Subjective assessment of nuclear purity did not vary between samples subjected to the same sucrose conditions. Purity varied inversely with nuclear yield as the column height was increased, for both 1.8 and 2.0 M sucrose. The low nuclear yield despite excellent purity using 5 ml of 2.2 M sucrose precluded use of this sucrose concentration. The highest nuclear yields were observed with either 5 ml or 10 ml of 1.8 M sucrose. Since nuclear purity was substantially better with 10 ml as opposed to 5 ml of 1.8 M sucrose, all further experiments involving nuclear purification were done using 10 ml of 1.8 M sucrose.

In order to determine the optimal method for preparation of tissue homogenate prior to nuclear purification, pulverization under liquid nitrogen was compared to no pulverization, followed by a comparison of Polytron and glass homogenization, as outlined in Figure 1. The results are presented in Table VII, and represent the means of 3 experiments on different BPH specimens. Omitting pulverization from tissue preparation resulted in a significant loss of AR_N. Although glass homogenization resulted in a higher nuclear yield than Polytron homogenization, it was accompanied by a greater degree of nuclear impurity. When AR_N was adjusted for nuclear recovery variations by expression as fmol/mg nuclear DNA, there was little difference between Polytron and glass homogenization.

TABLE VI

Studies of Variations in Sucrose Sedimentation Methods During Nuclear Purification for BPH

	Concentration of Sucrose					
	1.8 M			2.0 M		2.2 M
Sucrose Solution Volume	5 ml	10 ml	20 ml	5 ml	10 ml	5 ml
Nuclear Recovery * (percent)	75	69	42	55	47	11
Nuclear Purity †	impure	moderately pure	pure	moderately pure	pure	pure

* Recovery = $\frac{\text{post-sucrose DNA concentration}}{\text{pre-sucrose DNA concentration}} \times 100$

† Assessed by 1 observer using light microscopy

TABLE VII

Studies of Variations in Pulverization and Homogenization Techniques for Nuclear Purification for BPH

Method of Preparation	Nuclear Recovery § (percent)	Nuclear Purity †	AR_N * Total
Polytron only	36	pure	1404
Pulverization, Polytron	33	pure	3709
Pulverization, glass homogenizer	64	impure	3480

* All values expressed in fmol/mg nuclear DNA. Mean of 3 experiments.

§ Recovery = $\frac{\text{post-sucrose DNA concentration}}{\text{pre-sucrose DNA concentration}} \times 100$

† Relative nuclear purity assessed by 1 observer using light microscopy

Studies of Nuclear Androgen Receptor Concentrations in Crude and Purified Preparations of Benign Prostatic Hyperplasia

In order to determine the optimal method of measuring AR_N in the prostate, extractable, non-extractable (in crude preparations), and matrix-bound (in purified preparations) AR_N was quantified in 3 BPH specimens, as outlined in Figure 2. The results of these studies are presented in Table VIII, with all values representing means of the 3 experiments. When observed binding is corrected for variable nuclear recovery, by expression in fmol/mg nuclear DNA, very little difference is observed for AR concentrations in crude and purified nuclear preparations.

TABLE VIII

Studies of AR_N in Crude and Purified Preparations of Nuclei of BPH *

Method of Preparation	Method of Extraction	AR_N Concentration (fmol/mg nuclear DNA)			
		Extractable	Non-extractable	Matrix-bound	Total
Crude	None	-	-	-	231
	KCl	78	247	-	325
	KCl/DNase	44	161	-	205
Pure	KCl	18	318	-	336
	KCl/DNase	49	-	279	328

* All values represent mean of 3 experiments

CHAPTER IV

DISCUSSION

Benign Prostatic Hyperplasia

The role of ER in the development of BPH is currently unresolved. If ER has a significant role in this disorder, it should be uniformly detectable in BPH specimens. Auf and Ghanadian (1982) and Donnelly et al (1983) have presented the most reliable studies to date on ER in BPH. In the former study, ER_C and extractable ER_N were detected in 94 percent of BPH specimens, but the binding affinity was considerably lower than that usually reported for ER. In the latter study, despite the use of sodium molybdate, PMSF, and Scatchard analysis, ER_C could be demonstrated in only 53 percent of assayed specimens. Furthermore, ER_C was only present in low concentrations, implying that it might be less important than AR_C or PgR_C, both of which were present in greater than twice the concentration of ER_C in the same study. We therefore wished to extend these investigations by quantitating ER_N in BPH, by employing a heat exchange to determine whether endogenous estrogens were occupying ER and therefore preventing its detection, and by determining whether type II estrogen binding sites were present in BPH.

The methods used in quantitating ER_N (extractable and matrix-bound) were identical to those which had been successful in measuring AR_N in BPH (Donnelly, 1982). However, ER_N was not detectable in any of the BPH specimens investigated (n=9), or in the two normal specimens assayed for comparison. Since some of the specimens had been in storage at -70°C for several months, it was postulated that receptor degradation might have occurred. However, ER_N was also not detectable in subsequently assayed fresh BPH specimens.

Previous investigations in our laboratory (Tildesley and McBlain, 1983) have demonstrated that a 30°C, 30 minute temperature exchange is an effective method of revealing ER_C occupied by endogenous estrogens in human mammary tumours. The temperature increases the rate of dissociation of endogenous steroid from the receptor, thereby allowing radioligand to bind to the receptor in its place. Our attempts to produce a similar steroid exchange in BPH have been unsuccessful, both for ER_C and ER_N. It is possible that different conditions of heating would be more effective in the prostate. However, since there was also absolutely no change in measureable binding using a 30°C, 30 minute exchange, this is unlikely. Alternative methods of steroid exchange such as treatment with 0.5 M sodium thiocyanate or 5 mM mersalyl acid (Tildesley and McBlain, 1983) have not yet been investigated in BPH.

Wilson et al (1975) have strongly implicated elevation of serum estradiol in the pathogenesis of canine BPH. Since estrogens generally act via a receptor-mediated pathway, it is reasonable to assume that ER would be present in human BPH if estrogens were a significant factor in this disorder. Our investigations do not support the hypothesis that type I ER is important in BPH. However, it is possible that estrogens act through type II ER, as described by Clark et al (1976, 1978).

While this manuscript was in preparation, Ekman et al (1983) reported the presence of type I and type II ER_C and ER_N (salt-extractable and salt-resistant) in normal, hyperplastic, and malignant prostate specimens. Our preliminary investigations of type I and type II ER_C in the rat uterus have not revealed type II ER_C despite a variety of experimental conditions. Since demonstration of type II ER_C in the animal model used by the original investigators has as yet been unsuccessful, we have not attempted to measure type II ER_C in human prostate tissue.

In summary, although the finding of ER_C in some BPH specimens by Donnelly et al (1983) suggests a role for estrogen in BPH, we have been unable to demonstrate ER in the nuclei of BPH, or to detect endogenously occupied ER by steroid exchange assay. ER_N may indeed be present, but in low concentrations which are not detected by our assay, or the ER may be unstable and therefore inactivated by the vigorous nature of the nuclear isolation and extraction procedures. Either the magnesium of the nuclear purification buffer or the KCl of the extraction buffer may have inactivated any ER_N present.

It is also possible that ER_N is present in substantial amounts, but cannot be detected by addition of radioligand. Studies in the estrogen-sensitive human breast cancer MCF-7 cell line have shown that following translocation of ER_C to the nucleus, the steroid-receptor complex cannot be detected possibly due to modification of the receptor, or its binding sites (Horwitz and McGuire, 1978a and 1980). This phenomenon has been termed "nuclear processing" of receptor, and may represent an equilibrium between degradation and synthesis, or a redistribution of receptor within nuclear binding sites of different affinities (Horwitz and McGuire, 1978b). Alternatively, ER_N may be unimportant in BPH, in which case the DHT accumulation associated with BPH may simply be due to an increased stromal 5α-reductase activity, as suggested by McLoughlin et al (1983).

Adenocarcinoma of the Prostate

While the presence or concentration of AR_C does not correlate with therapeutic response in adenocarcinoma of the prostate (Wagner and Schulze, 1978; de Voogt and Dingjan, 1978; Martelli et al, 1980; de Vere White and Olsson; Ekman, 1982), it has been suggested that the concentration of AR_N may provide a better prognostic index for this disease (de Vere White and Olsson, 1981; Ekman, 1982). Two recent reports suggest a correlation between extractable AR_N and hormone responsiveness in patients with prostatic cancer (Mohla et al, 1982; Trachtenberg and Walsh, 1982). Since most prostatic cancers are AR-positive, a critical level of AR binding may be a more appropriate index than simply the presence or absence of receptor. Mohla et al (1982) reported that 86 percent of patients with an extractable AR_N concentration greater than 50 fmol/g of tissue responded favourably to endocrine therapy, while only 28 percent of those with an AR_N concentration less than 50 fmol/g of tissue responded favourably. Trachtenberg and Walsh (1982) established their critical concentration of extractable AR_N with regard to duration of response at 110 fmol/mg DNA, however, there was considerable overlap in receptor concentration between responders and nonresponders. Furthermore, the use of a crude nuclear pellet in this study could have resulted in cytoplasmic contamination, and therefore, any observed binding might have been to cytoplasmic as well as nuclear receptor. To avoid binding to residual cytoplasmic components, we have used a relatively pure nuclear preparation for our assays. Our results for extractable AR_N suggest that this receptor assay is a useful prognostic index, in accordance with Mohla et al (1982) and Trachtenberg and Walsh (1982), even though the patient data in the latter study does not entirely support this conclusion.

In previous investigations in our laboratory (Donnelly, 1982), nuclear matrices were isolated from normal and hyperplastic prostate specimens, and shown to contain significant quantities of AR_N (see Table IX and Figure 13). Barrack and Coffey (1980) have demonstrated that, in the rat prostate, nuclear matrix-bound AR is the primary determinant of androgen action. Therefore, we decided to investigate matrix-bound AR_N in prostate cancer. Our results indicate that although the concentration of matrix-bound AR_N and extractable AR_N are useful prognostic indices, ($p < 0.05$) total nuclear AR is the major determinant of androgen dependency in prostatic adenocarcinoma ($p < 0.02$). Since 75 percent of those patients with no detectable extractable AR_N had significant levels of matrix-bound AR_N in our series, combined quantification of extractable and matrix-bound AR_N is necessary for accurate prognostication.

Due to our relatively small sample size, we are currently unable to establish a critical concentration of matrix-bound AR_N to reliably predict tumour androgen dependency. However, only one patient with matrix-bound AR_N concentration greater than 200 fmol/g of tissue had disease progression, and only one patient with matrix-bound AR_N concentration less than 100 fmol/g of tissue had disease regression. Therefore, matrix-bound AR_N concentrations greater than 200 fmol/g of tissue or less than 100 fmol/g of tissue appear to correlate with disease regression or progression, respectively. Matrix-bound AR_N concentrations between 100 and 200 fmol/g of tissue are of little predictive value, as 3 of these patients had disease progression and 2

Table IX

Androgen Receptor of the Prostate †

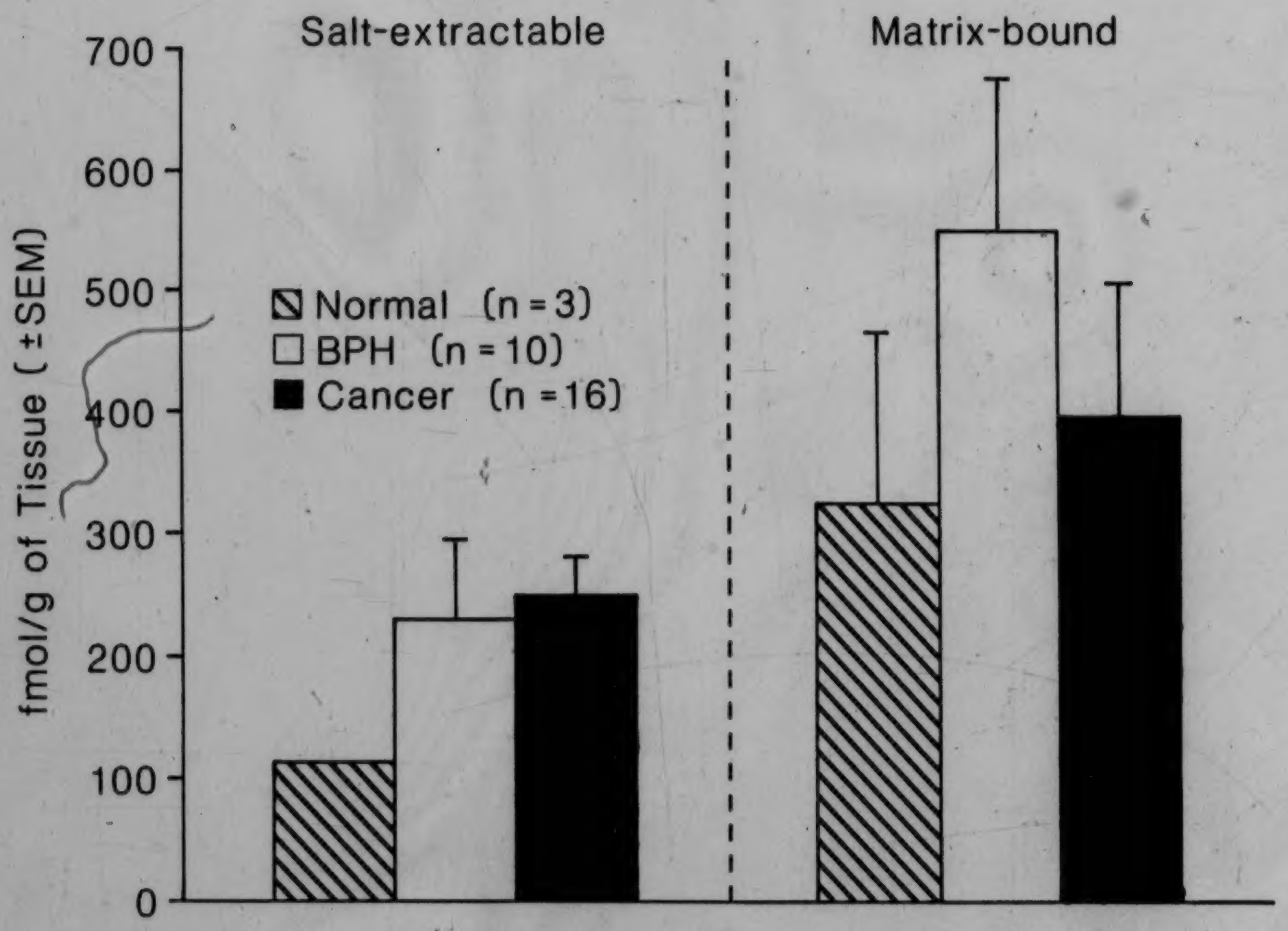
Tissue	n	AR_C	AR_N Extractable	AR_N Matrix-bound	AR Total
Normal**	3	606 ± 59	113*	325 ± 139	1044
BPH**	10	595 ± 59	230 ± 65	548 ± 129	1373
Cancer	16	1028 ± 255	251 ± 31	396 ± 112	1675

† Mean ± S.E.M. for AR-positive samples, expressed in fmol/g of tissue

* Only 1 of the 3 tissues exhibited specific binding of $[^3H]$R1881

** Data from Donnelly (1982)

Figure 13. Nuclear androgen receptor of the prostate. In both salt-extractable and matrix-bound compartments, hatched bars = normal prostate (n=3), open bars = BPH (n=10), dark bars = prostate cancer (n=16). Bar height represents mean AR_N concentration (fmol/g of tissue) ± S.E.M., for positive cancer specimens only (all normal and BPH specimens were positive for AR).


Salt-extractable
Matrix-bound
fmol/g of Tissue (±SEM)
700
600
500
400
300
200
100
0
▨ Normal (n = 3)
☐ BPH (n = 10)
■ Cancer (n = 16)

had disease regression. We are also currently unable to predict relapse rates during hormonal therapy, due to our short period of observation. However, these data will be forthcoming with continued observation.

Previous quantification of AR in normal and hyperplastic prostates in our laboratory (Donnelly, 1982) permits comparisons of AR concentrations among the various tissue types (see Table IX and Figure 13). AR_C represented 43-61 percent of total cellular AR in the three types of tissue; extractable AR_N represented 10-16 percent; and matrix-bound AR_N represented 22-39 percent. Matrix AR_N therefore represented 74, 70, and 61 percent of total AR_N in normal, hyperplastic and malignant tissue, respectively (data derived from Table IX). As the number of normal tissue specimens is very small, no statistical analyses were done comparing the concentrations of AR in normal tissue with those found in BPH or cancer. The unpaired Student's t-test reveals that the observed differences in all AR values between BPH and cancer are insignificant ($p > 0.05$). The relative concentrations of AR_N are depicted in histogram form in Figure 13, which illustrates the marginal differences between normal, hyperplastic and malignant tissue, in both extractable and matrix-bound receptor compartments. These results have not been corrected for any loss of nuclei during nuclear purification.

While this work was in progress, Barrack et al (1983) reported a series of 11 prostate cancer specimens in which AR_N was quantified as either salt-extractable or salt-resistant (see below). However, these investigators used a crude homogenate (which would contain considerable cytoplasmic debris), rather than a purified preparation (Donnelly, 1982). In contrast, as mentioned above, the use of relatively pure nuclei for the study reported herein gives considerable confidence that the steroid binding detected was binding to nuclear components, and not to any residual cytoplasmic components.

As shown in Table X, Barrack et al (1983) expressed their results as fmol/mg DNA, thus correcting for any loss of nuclei. However, they did not correlate AR_N concentrations with patient response to hormonal therapy. Although the follow-up period of six months for our patients is relatively short, androgen dependency and resultant benefit from androgen suppression frequently only lasts several months. Therefore we feel justified in concluding that evidence of disease regression at six months on hormonal therapy is indicative of androgen dependency as predicted by extractable, matrix-bound, or preferably, total AR_N.

The Problem of Expression of Nuclear Androgen Receptor Concentration in Prostate Cancer

In order to provide a meaningful quantification of AR, it was necessary to adopt the most accurate assay methods, and in addition, to determine of the most appropriate mode of expression of any measured binding.

Although single point microassays have been advocated for estimation of steroid receptor content (Hicks and Walsh, 1979; Barrack et al, 1982), the most reliable method remains saturation assays with data analysis by the method of Scatchard (1949), which provides the maximal binding capacity (B max), dissociation constant (K_d), and correlation coefficient (r) (Murphy et al, 1980; Ekman, 1982).

B max is inconsistently expressed as fmol/mg protein, fmol/mg DNA, or fmol/g of tissue, by various investigators, making comparisons among studies difficult. Any of these methods is suitable for benign tissue,

Table X

Comparison of Prostate Cancer AR_N Concentrations Expressed as fmol/mg DNA for Existing Studies

Author	salt-extractable	salt-resistant	total nuclear
Trachtenberg and Walsh (1982)* (n = 23)	207	not determined	not determined
Barrack et al (1983)‡ (n = 11)	227	128	355
Gonor (1983)† (n = 8)	113	336	449

† Salt-resistant = Matrix-bound in present series. Expressed in fmol/mg nuclear DNA.

* Expressed as fmol/starting DNA equivalent.

‡ Expressed as fmol/mg DNA.

which has a relatively constant DNA concentration of 1-2 mg DNA/g of tissue (Hicks and Walsh, 1979; Sirett and Grant, 1982). However, in the malignant gland, the DNA content is highly variable due to extreme degrees of aneuploidy (Zetterberg and Esposti, 1976; Coffey and Isaacs, 1981b), which could render this mode of expression unsuitable.

In our series of prostatic cancer samples, DNA concentrations ranged from 0.2-15.9 mg of DNA/g of tissue (mean value 5.3 mg of DNA/g of tissue) while Ekman et al (1979) found 1.3-14.0 mg of DNA/g of tissue (mean value 5.4 mg of DNA/g of tissue). Thus, specimens containing identical amounts of receptor could have vastly different AR_N concentrations when expression is based on the DNA concentration.

It has even been suggested that the greater the ploidy, the worse the response to hormonal therapy (Bohm and Sandritter, 1966). Since an increased ploidy results in a greater DNA concentration, with less resultant androgen binding when expressed per mg of DNA, AR_N values expressed per mg DNA will be inversely related to the degree of aneuploidy. Therefore, in prostate cancer, a low AR_N concentration (expressed per mg of DNA) indicates high ploidy and a resultant poor response to hormonal therapy possibly on this basis alone. It is thus evident that progression of disease in the presence of a low AR_N concentration (expressed per mg of DNA) may be correlated with aneuploidy, which in turn produces the artifactually low AR_N concentration.

In view of these difficulties, we propose that in malignant tissue, AR_N concentrations should be expressed in fmol/g of tissue to avoid ploidy-dependent receptor concentrations. Although results for cytoplasmic receptor assays may be expressed in fmol/mg of cytosol protein, this is less appropriate for nuclear assays, where the significance of protein concentrations is unknown.

The expression of binding capacity per mg of DNA becomes even more problematic when nuclear purification is introduced. The inevitable loss of nuclei during nuclear purification produces a lower DNA concentration in the purified preparation. Binding may therefore be expressed either per mg of starting DNA, or per mg of nuclear DNA, the latter expression adjusting for variable nuclear recovery. When data are expressed per mg of nuclear DNA, the value obtained is therefore higher than when expressed per mg of starting DNA. In order to correct for nuclear loss while avoiding the problem of aneuploidy, the binding must be expressed as fmol/g of tissue (corrected), which is obtained by dividing the binding in fmol/g of tissue by the percentage nuclear recovery. This value represents the amount of binding present per g of tissue, corrected for nuclear loss during purification, and is the most logical mode of expression for prostate cancer if one is assuming variable nuclear recovery, since any mode of expression which involves DNA concentration is unsuitable because of the wide range of ploidy.

Although Mohla et al (1982) express their results for extractable AR_N in prostate cancer in fmol/g of tissue, Trachtenberg and Walsh (1982) rely on fmol/mg starting DNA equivalent. This latter expression is calculated by determining the DNA concentration in the initial tissue homogenate and using this starting DNA value to express the concentration of AR extracted from the resulting crude nuclear pellet. This derivation assumes a uniform nuclear DNA concentration, which is not present in adenocarcinoma of the prostate. To allow comparison with other studies, we have expressed our cancer results per mg of nuclear

DNA (see Table X). It is apparent from this table that we observed comparable extractable AR_N to that seen by other investigators, but that our matrix-bound AR_N was significantly higher.

It is noteworthy that for studies of benign tissue, DNA concentrations remain useful, and have the advantage of allowing correction for variable nuclear recovery (when expressed per mg nuclear DNA) thereby permitting comparison of binding in crude and purified preparations.

Nuclear Purification and Receptors

In the quantification of any nuclear receptor, a basic prerequisite is that the observed binding must be to nuclear components, rather than to cytoplasmic or extracellular contaminants. Nuclear purification is therefore a logical measure in quantification of nuclear receptors in the prostate. For the prostate cancer AR_N studies presented above, we relied on the basic method of nuclear purification utilized by Donnelly (1982). We have since attempted to refine this technique by investigating three aspects of nuclear purification: (1) determination of the optimal sucrose concentration and volume, (2) determination of the optimal method of tissue homogenization, (3) comparison of measureable AR_N under conditions of crude and purified nuclear preparations.

Our studies of variations in sucrose sedimentation (see Table VI) indicate that an optimal balance between nuclear purity and nuclear recovery is obtained with 10 ml of 1.8 M sucrose. Nuclear recovery is enhanced marginally with 5 ml of 1.8 M sucrose, at the expense of a substantial loss of purity. Similarly, nuclear purity is enhanced with

20 ml of 1.8 M sucrose, at the expense of a substantial loss of nuclei. Increasing the concentration of sucrose to 2.0 M produces a relative increase in nuclear purity, but with low nuclear recovery. Therefore, all subsequent nuclear purifications were done using 10 ml of 1.8 M sucrose.

Periodic evaluation of cancer preparations with light microscopy demonstrated variable cytoplasmic contamination. Since a glass homogenizer was used for these preparations, it was necessary to determine whether cytoplasmic contamination might be lessened, while simultaneously maintaining reasonable nuclear recovery, using an alternative method of tissue homogenization. An additional parameter examined in these experiments was the need for tissue pulverization under liquid nitrogen prior to homogenization. Our routine method of pulverization by Thermovac compression gun or mortar and pestle produced a fine powder which we felt was more uniform in consistency than simply chopping the tissue with a razor blade. Results of these studies (see Table VII) indicate that pulverization is essential to avoid underestimation of AR_N in BPH. Furthermore, although nuclear recovery is superior with glass homogenization, this is associated with a marked impurity. When AR_N concentrations are expressed in fmol/mg nuclear DNA, thereby correcting for nuclear loss, Polytron and glass homogenization produce similar measureable quantities of AR_N. Because of the comparable AR_N detection, with much better purity, tissue pulverization followed by Polytron homogenization is thus the preferred method of nuclear preparation in BPH.

Other investigators of salt-resistant AR_N (Barrack et al, 1983) have relied on a crude nuclear preparation without purification (see above). To determine whether the differences in AR_N concentration observed between our series and that of Barrack et al (1983) (see Table X) were due to uncovering of binding sites, it was essential to compare AR_N measured in crude and purified preparations of BPH. These studies have demonstrated that salt-resistant or matrix-bound AR_N concentrations are similar in crude and purified BPH preparations, when corrected for nuclear loss with purification. Therefore, our higher levels of matrix-bound AR_N in prostate cancer specimens apparently cannot be explained by the unmasking of binding sites by nuclear purification. Barrack et al (1983) used TEG (Tris-EDTA-glycerol) buffer for their subcellular fractionation procedures, while STM (Sucrose-Tris-magnesium) buffer was used in our series. STM contains 5 mM $MgSO_4$ which is a known stabilizer of nuclear membranes (Berezney and Coffey, 1977), and omission of $MgSO_4$ may explain the wide range of nuclear recoveries (averaging 70 percent) observed by Barrack et al (1983). If we express our results in fmol/mg starting DNA, thereby discounting nuclear loss during purification, the mean matrix-bound AR_N concentration is 148 fmol/mg starting DNA (see Table 1), comparable to that observed by Barrack et al (1983). It is possible that by not correctly accounting for loss of nuclei during tissue preparation, these investigators may not have accurately quantified AR_N concentrations.

It must be emphasized that if results are expressed in fmol/g of tissue, nuclear purification produces lower AR_N values, due to nuclear loss. Therefore, for BPH specimens, comparison between crude and purified preparations requires quantification of nuclear recovery and expression of binding in fmol/mg nuclear DNA. Alternatively, expression of AR_N concentration in fmol/g of tissue (corrected) will also allow comparison between crude and purified preparations.

The Future

We have demonstrated the utility of extractable, matrix-bound, and total AR_N quantifications in adenocarcinoma of the prostate for prediction of androgen dependency, and determined that the optimal mode of expression for AR_N in prostate cancer specimens is fmol/g of tissue, corrected for nuclear recovery in purified preparations. Our preliminary studies do not confirm the role of ER_N in the pathogenesis of BPH, and we have been unable to demonstrate type II ER_C in the immature rat uterus. We have therefore been unable to apply this concept to the prostate. We have also refined the methods of nuclear purification for prostate specimens, and demonstrated the equivalence of crude and purified preparations in quantifications of AR_N.

Future considerations for the application of steroid receptor assays in prostate cancer include dissociation of AR_N from the nuclear matrix, determination of the effect of sodium molybdate on AR_N, more extensive patient follow-up, and determination of a critical concentration of matrix-bound AR_N necessary for androgen dependency. Furthermore, ER_N and nuclear PgR (PgR_N) have not been investigated in prostate cancer.

In benign prostatic hyperplasia, our findings regarding ER_C and ER_N must be investigated further, possibly by determination of the presence or absence of type II sites within the prostate, or alternate methods of exchange assay, in order to clarify the role of estrogen and ER in the pathogenesis of BPH. In addition, PgR_N has yet to be quantified in BPH, and this receptor may also modulate prostatic cellular growth.

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APPENDICES

APPENDIX I

NATIONAL PROSTATIC CANCER PROJECT RESPONSE CRITERIA

(a) Objective Complete Response (all of the following)

1. Tumour masses, if present, totally disappeared and no new lesions appeared.
2. Elevated acid phosphatase, if present, returned to normal.
3. Osteolytic lesions, if present, recalcified.
4. Osteoblastic lesions if present, normalized.
5. If hepatomegaly is a significant indicator there must be a complete reduction in liver size, and normalization of all pre-treatment abnormalities of liver function.
6. No significant cancer-related deterioration in weight (<10%), symptoms, or performance status (became or remained ambulatory).

(b) Objective Partial Regression (all of the following)

1. At least one tumour mass, if present, is reduced by 50% in x-sectional area.
2. Elevated acid phosphatase, if present, returned to normal.
3. Osteolytic lesions, if present, do not surpass.
4. Osteoblastic lesions, if present, do not progress.
5. If hepatomegaly is a significant indicator, there must be a reduction in liver size and at least a 30% improvment of all pre-treatment abnormalities of liver function.
6. There may be no increase in any other lesion and no new areas of malignant disease may appear.

7. No significant cancer-related deterioration in weight (<10%), symptoms, or performance status (improved or remained the same).

(c) Objective Stable (all of the following)

1. No new lesions occurred and no lesions measurably present increased more than 25% in x-sectional areas.
2. Acid phosphatase level decreases, though need not return to normal.
3. Osteolytic lesions, if present, do not appear to worsen.
4. Osteoblastic lesions, if present, remain stable.
5. Hepatomegaly, if present, does not worsen by more than 30% and symptoms of hepatic abnormalities do not worsen.
6. No significant cancer-related deterioration in weight (<10%), symptoms, or performance status (improved or remained the same).

(d) Objective Progression (any of the following)

1. Significant cancer-related deterioration in weight (<10%), symptoms, or performance status (at least one score level).
2. Appearance of new areas of malignant disease.
3. Increase in any previously measureable lesion greater than 25% in x-sectional area.
4. Development of recurring anemia secondary to prostate cancer.
5. Development of urethral obstruction.

NOTE: An increase in acid or alkaline phosphatase alone is not to be considered an indication of progression, and should be used only in conjunction with other criteria.

APPENDIX 2

Pre-Treatment Patient Data

Patient No.	Hospital No. #	Age	Stage	Grade*	Previous RT**	Bone Scan†	Wt. (kg)	Hgb (gm %)	PAP§ (IU/l)	Bone Pain
1	5395165	75	D2	4	nil	(+)	70	11.2	3.7	(+)
2	6080006	78	D2	5	nil	(+)	63	11.1	5.0	(+)
3	6094619	72	D2	4	nil	(+)	75	14.4	13.3	(+)
4	3840501	78	C	5	1977	(-)	73	15.9	0.4	(-)
5	4086211	74	C	3	1976	(-)	63	12.5	1.2	(+)
6	6256754	70	D2	3	nil	(+)	66	12.8	0.6	(-)
7	3139169	63	D2	5	nil	(+)	75	12.5	0.3	(-)
8	6067268	71	D2	4	nil	(+)	84	12.2	26.0	(+)
9	3906625	75	D1	4	nil	(-)	73	14.2	1.6	(-)
10	0714816	70	D2	4	nil	(+)	69	12.2	5.3	(-)
11	6074793	68	D2	5	nil	(+)	61	10.6	8.6	(+)
12	6087506	85	D2	5	nil	(+)	80	8.6	16.9	(-)
13	3488202	78	D	4	nil	(+)	70	15.3	2.9	(-)
14	6155287	68	C	3	nil	(-)	92	16.2	0.5	(+)
15	6180566	70	D2	5	nil	(+)	77	13.4	0.6	(+)
16	162404	75	D	4	1977	(+)	69	9.7	16.0	(-)

Hospital No. of patients 1 - 15 = University of Alberta Hospital; patient 16 = Charles Camsell Hospital

* Grading of all tumours was by the Gleason system. Where major and minor patterns were detected, only major pattern is denoted here. ** RT = Radiotherapy

† Bone Scan assessments: (+) metastases; (-) no metastases

§ PAP = prostatic acid phosphatase concentration

APPENDIX 3

Post-Treatment Patient Data

Patient No.	Therapy†	Interval (months)	Survival‡	Bone Scan**	Wt. (kg)	Hgb (gm %)	PAP§ (IU/L)	Bone Pain	Appetite	Ambulation	Disease‡‡ Response
1	(O)	9	alive	(+)(↑)	70	12.8	11.1	(+)	worse	worse	progression
2	(S)	5	died @ 5m	(*)	(*)	(*)	(*)	(*)	(*)	(*)	death
3	(O)	7	alive	(+)(↓)	85	13.3	0.3	(-)	same	improved	partial regression
4	(S)	7	died @ 7m	(*)	(*)	(*)	(*)	(*)	(*)	(*)	death
5	(S)	6	alive	(-)	83	13.0	0.5	(-)	same	same	stable
6	(S)	6	alive	(+)	63	9.3	0.5	(+)	same	worse	progression
7	(S)	4	died @ 4m	(*)	(*)	(*)	(*)	(*)	(*)	(*)	death
8	(S)	1	died @ 1m	(*)	(*)	(*)	(*)	(*)	(*)	(*)	death
9	(S)	14	alive	(-)	73	12.4	0.7	(-)	same	same	partial regression
10	(O)	14	alive	(+)(↑)	69	10.6	2.0	(+)	same	same	progression
11	(O)	14	alive	(+)(↓)	63	12.2	1.7	(-)	same	improved	stable
12	(S)	13	alive	(+)(↓)	90	10.6	0.9	(-)	same	same	partial regression
13	(S)		alive	(-)	72	14.9	1.5	(-)	same	same	stable
14	(S)	10	alive	(*)	96	14.5	1.0	(-)	same	same	stable
15	(S)	10	alive	(-)	78	13.0	0.5	(-)	same	same	complete response
16	(O)	6	alive	(-)	67	13.8	3.2	(-)	improved	same	stable

‡ time in months (m) of survival after treatment initiated

** bone scan assessments: (+) metastases; (-) no metastases; (↓) improved; (↑) worsened; (*) not obtained

† (O) orchidectomy; (S) stilbestrol 3 mg by mouth once daily

§ PAP = prostatic acid phosphatase concentration

‡‡ see Appendix 1

APPENDIX 4

Patient Androgen Receptor Data (Adenocarcinoma of the Prostate)

	AR_C				AR_N (Extract 1)					
Patient No.	A	B	F	G	A	C	D	E	F	G
1	1439	41	0.96	0.3	106	-	-	-	0.75	0.5
2	455	34	0.83	0.3	37	-	-	-	0.91	0.2
3	1035	48	0.97	0.4	0	0	0	0	-	-
4	0	0	0	0	0	0	0	0	-	-
5	0	0	0	0	213	36	58	349	0.74	4.4
6	1336	297	0.74	0.9	0	-	-	-	-	-
7	149	8	0.85	0.2	56	77	128	93	0.80	1.3
8	232	9	0.87	0.1	73	-	-	-	0.84	0.4
9	923	18	0.76	0.1	253	26	46	460	0.95	0.5
10	414	23	0.79	0.3	0	-	-	-	-	-
11	967	31	0.94	0.1	246	-	-	-	0.81	1.0
12	710	22	0.93	0.1	231	55	123	525	0.95	0.8
13	755	24	0.84	0.4	90	-	-	-	0.90	1.7
14	385	11	0.80	0.2	46	47	78	177	0.78	0.2
15	1660	61	0.79	1.1	? (spilled)					
16	3938	140	0.96	0.2	190	-	-	-	0.87	1.1

A = fmol/g of tissue
B = fmol/mg of cytosol protein
C = fmol/mg starting DNA
D = fmol/mg nuclear DNA
E = fmol/g of tissue (corrected)
F = r value (correlation coefficient)
G = K_d (nM) (dissociation constant)

APPENDIX 4 - continued

Patient Androgen Receptor Data (Adenocarcinoma of the Prostate)

	AR_N (Extract 2)						AR_N (Extracts 1 & 2)			
Patient No.	A	C	D	E	F	G	A	C	D	E
1	0	-	-	-	-	-	106	-	-	-
2	0	-	-	-	-	-	37	-	-	-
3	0	0	0	0	-	-	0	0	0	0
4	0	0	0	0	-	-	0	0	0	0
5	0	0	0	0	-	-	213	36	58	349
6	0	-	-	-	-	-	0	-	-	-
7	0	0	0	0	-	-	56	77	128	93
8	0	-	-	-	-	-	73	-	-	-
9	88	9	16	160	0.72	0.5	341	35	62	620
10	0	-	-	-	-	-	0	-	-	-
11	62	-	-	-	0.96	4.2	308	-	-	-
12	0	0	0	0	-	-	231	55	123	525
13	0	-	-	-	-	-	90	-	-	-
14	0	0	0	0	-	-	46	47	78	177
15	184	24	35	270	0.98	0.3	184	24	35	270
16	0	-	-	-	-	-	190	-	-	-

A = fmol/g of tissue
B = fmol/mg of cytosol protein
C = fmol/mg starting DNA
D = fmol/mg nuclear DNA
E = fmol/g of tissue (corrected)
F = r value (correlation coefficient)
G = K_d (nM) (dissociation constant)

APPENDIX 4 - continued

Patient Androgen Receptor Data (Adenocarcinoma of the Prostate)

	AR_N (Matrix)									AR_N (Total)AR	
								(Total)			
Patient #	A	C	D	E	F	G	A	C	D	E	A
1	151	-	-	-	0.97	1.0	257	-	-	-	1696
2	71	-	-	-	0.92	0.7	108	-	-	-	563
3	229	14	30	487	0.82	0.3	229	14	30	64	1264
4	0	0	0	0	-	-	0	0	0	0	0
5	96	16	26	157	0.73	0.7	309	52	84	137	309
6	118	-	-	-	0.94	1.0	118	-	-	-	1454
7	0	0	0	0	-	-	56	77	128	213	205
8	246	-	-	-	0.91	0.8	319	-	-	-	478
9	1330	134	242	2418	0.99	0.7	1671	169	304	553	2594
10	128	-	-	-	0.99	1.1	128	-	-	-	542
11	375	-	-	-	0.99	0.6	683	-	-	-	1650
12	702	167	373	1595	0.96	0.6	933	222	496	1127	1643
13	197	-	-	-	0.88	1.1	287	-	-	-	1042
14	449	228	860	1727	0.72	1.7	495	275	938	3607	880
15	1281	331	485	1883	0.97	1.0	1465	355	520	764	3125
16	164	-	-	-	0.92	0.5	354	-	-	-	4292

A = fmol/g of tissue
B = fmol/mg of cytosol protein
C = fmol/mg starting DNA
D = fmol/mg nuclear DNA
E = fmol/g of tissue (corrected)
F = r value (correlation coefficient)
G = K_d (nM) (dissociation constant)

APPENDIX 5

Patient Progesterone and Estrogen Receptor Data
(Adenocarcinoma of the Prostate)

	PgR_C				ER_C			
Patient #	A	B	C	D	A	B	C	D
1	596	17	0.92	0.9	0	0	-	-
2	134	10	0.97	0.3	0	0	-	-
3	1230	57	0.92	0.5	0	0	-	-
4	451	24	0.66	0.7	0	0	-	-
5	0	0	-	-	0	0	-	-
6	742	165	0.90	0.3	153	34	0.65	0.1
7	0	0	-	-	0	0	-	-
8	346	14	0.55	0.1	175	7	0.74	0.4
9	1292	25	0.82	1.0	386	7	0.56	0.1
10	0	0	-	-	0	0	-	-
11	0	0	-	-	0	0	-	-
12	3253	101	0.92	1.8	301	9	0.94	0.2
13	861	28	0.99	0.1	387	12	0.82	1.0
14	700	20	0.70	0.7	0	0	-	-
15	0	0	-	-	268	10	0.52	0.7
16	788	28	0.92	3.9	0	0	-	-

A = fmol/g of tissue
B = fmol/mg of cytosol protein
C = r value (correlation coefficient)
D = K_d (nM) (dissociation coefficient)

APPENDIX 6

Steroid Dilutions for Saturation Analysis

Solution A

R1881: 37.5 μl 1000 nM $[^3\text{H}]$R1881 + 37.5 μl (1 mM) TA*** + 675 μl buffer
Estradiol: 85.2 μl 440 nM $[^3\text{H}]$17β-estradiol + 665 μl buffer
R5020: 85.2 μl 440 nM $[^3\text{H}]$R5020 + 649 μl buffer + 8.5 μl (44 μM) Dexamethasone + 8.5 μl (44 μM) DHT

	$[^3\text{H}]$ Steroid	Cold Steroid**	Buffer + 5.68% Ethanol	Initial Volume	Final Volume	Concentration (nM)
1.	264 μl of A	-	264 μl buffer	528 μl	300 μl	25
2.	228 μl of 1	-	342 μl *	570 μl	"	10
3.	270 μl of 2	-	270 μl *	540 μl	"	5
4.	240 μl of 3	-	240 μl *	480 μl	"	2.5
5.	180 μl of 4	-	270 μl *	450 μl	"	1
6.	150 μl of 5	-	150 μl *	300 μl	"	0.5
7.	216 μl of A	123 μl cold **	93 μl buffer	432 μl	"	25
8.	132 μl of 7	-	198 μl *	330 μl	"	10
9.	30 μl of 8	-	270 μl *	300 μl	"	1

* 170.4 μl Ethanol + 943.7 μl buffer
** 10 μl (440 μM) unlabelled steroid + 490 μl buffer (Estradiol and R5020)
** 4.4 μl (1 mM) unlabelled R1881 + 495.6 μl buffer (R1881)
*** 50 μl of 10 mM TA + 450 μl buffer.

APPENDIX 7

Steroid Dilutions for Expanded Saturation Analysis

	$[^3H]$ Estradiol	Cold Steroid***	Buffer + 5.68% Ethanol**	Initial Volume	Final Volume	Incubated Concentration (nM)
1.	217 μl of A*	-	219 μl buffer	436 μl	100 μl	40
2.	336 μl of 1	-	48 μl **	384 μl	"	35
3.	284 μl of 2	-	48 μl **	332 μl	"	30
4.	231 μl of 3	-	46 μl **	277 μl	"	25
5.	177 μl of 4	-	44 μl **	221 μl	"	20
6.	121 μl of 5	-	40 μl **	161 μl	"	15
7.	62 μl of 6	-	124 μl **	186 μl	"	5
8.	86 μl of 7	-	86 μl **	172 μl	"	2.5
9.	72 μl of 8	-	108 μl **	180 μl	"	1
10.	80 μl of 9	-	80 μl **	160 μl	"	0.5
11.	60 μl of 10	-	90 μl **	150 μl	"	0.2
12.	50 μl of 11	-	50 μl **	100 μl	"	0.1
13.	72 μl of A	5.8 μl of DES (0.5 mM)	66 μl buffer	144μl	"	40
14.	44 μl of 13	-	74 μl **	118 μl	"	15
15.	18 μl of 14	-	90 μl **	108 μl	"	2.5
16.	8 μl of 15	-	92 μl **	100 μl	"	0.2

* Solution A = 30 μl (4000 nM) $[^3H]$Estradiol + 270 μl buffer

** 75 μl Ethanol + 1425 μl buffer

*** 10 μl (5 mM) DES + 90 μl buffer

APPENDIX 8

Steroid Dilutions for Specificity Studies

Incubation No.	Volume of Matrix Suspension	[^{3}H] Steroid Added (Final Conc. = 2 nM)	Competitor Added**	Molar Excess of Competitor
1	200 µl +	25 µl (20 nM)[^{3}H] Steroid* +	25 µl buffer + 5% EtOH in buffer	0
2	" +	" " +	25 µl (0.04 µM) cold steroid***	2
3	" +	" " +	25 µl (0.2 µM) cold steroid	10
4	" +	" " +	25 µl (1 µM) cold steroid	50
5	" +	" " +	25 µl (2 µM) cold steroid	100
6	" +	" " +	25 µl (10 µM) cold steroid	500
7	" +	" " +	25 µl (20 µM) cold steroid	1000

* 20 µl (1000 nM) [^{3}H]R1881 + 20 µl (1 mM) TA + 912 µl buffer + 48 µl Ethanol.

** Cold Steroid Dilution: Make up ~ 2 ml of 5 mM stock i.e. ~ 2-3 mg steroid in a volume of Ethanol depending on the molecular weight of the steroid.

*** For 200 µM cold steroid: 80 µl (5 mM) cold steroid + 1.90 ml buffer + 20 µl Ethanol (5% Ethanol ÷ 10 = 0.5% Ethanol into final incubation

For 20 µM: 16.6 µl (200 µM) + 141.9 µl buffer + 7.5 µl Ethanol
For 10 µM: 66.3 µl (20 µM) + 63.0 µl buffer + 3.3 µl Ethanol
For 2 µM: 32.6 µl (10 µM) + 123.9 µl buffer + 6.5 µl Ethanol
For 1 µM: 63.0 µl (2 µM) + 59.8 µl buffer + 3.2 µl Ethanol
For 0.2 µM: 26.0 µl (1 µM) + 98.8 µl buffer + 5.2 µl Ethanol
For 0.04 µM: 30.0 µl (0.2 µM) + 114.0 µl buffer + 6.0 µl Ethanol